

Characterization of Two Site-Specific Mutations in Human Dihydrolipoamide Dehydrogenase Deteriorating the Apparent Enzyme Binding Affinities to Both Dihydrolipoamide and NAD⁺

Hakjung Kim

Department of Chemistry, College of Natural Science, Daegu University, Kyoungsan 38453, Korea.
E-mail: hjkim@daegu.ac.kr

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Dihydrolipoamide dehydrogenase (E3) (dihydrolipoamide: NAD⁺ oxidoreductase; EC 1.8.1.4) is a homodimeric flavoenzyme containing one FAD as a prosthetic group at each subunit.¹ E3 is a critical component in three α -keto acid dehydrogenase complexes (pyruvate, α -ketoglutarate, and branched-chain α -keto acid dehydrogenase complexes).² E3 catalyzes the reoxidation of the dihydrolipoyl prosthetic group attached to the lysyl residue(s) of the acyl-transferase components of the three α -keto acid dehydrogenase complexes. Because E3 is an essential component in the three α -keto acid dehydrogenase, a decrease in E3 activity can affect the activities of all three complexes. E3 deficient patients typically die at an early age because an E3 deficiency is a critical genetic defect that affects the central nervous system. This can result in Leigh syndrome with recurrent episodes of hypoglycemia and ataxia, permanent lactic acidemia and mental retardation.³

Along with glutathione reductase, thioredoxin reductase, mercuric reductase, and trypanothione reductase, E3 belongs to the pyridine nucleotide-disulfide oxidoreductase family.⁴ These enzymes consist of four structural domains (FAD, NAD, central, and interface domains).¹ They have similar catalytic mechanisms and structures. All of them have homodimeric structures containing an active disulfide center and a FAD in each subunit. Through the FAD and active disulfide center, this family catalyzes electron transfer between the pyridine nucleotides (NAD⁺ or NADPH) and their specific substrates.

Fig. 1 shows the sequence alignment of the Pro-154 and Pro-423 region of human E3 with the corresponding regions of E3s from various sources, such as pigs, yeast, *Escherichia coli*, and *Pseudomonas fluorescens*. Pro-154 is a component of a random coil structure between the short β -sheet structure F1, consisting of 3 amino acids, and the short

α -helix structure 4, composed of 4 amino acids. These structures are components of the NAD⁺ domain in human E3. Pro-154 is located close to NAD⁺ (Fig. 2(a)). Pro-423 is a part of the β -turn structure between the β -sheet structure G5, consisting of 6 amino acids, and α -helix structure 11, which is composed of 13 amino acids (Fig. 2(b)). These structures are components of the interface domain in human E3.

To examine the importance of Pro-154 and Pro-423 on the human E3 structure and function, the Pro-154 and Pro-423 were mutated site-specifically to Ala by site-directed mutagenesis. The following two questions were addressed using these site-specific mutations in human E3. First, to what extent is the structure and function of human E3

E3	Amino acid sequence
sp P09622 DLDH_HUMAN	154 L I A T G S E V T P F P G I T I D E D
sp P09623 DLDH_PIG	L I A T G S E V T P F P G I T I D E D
sp P09624 DLDH_YEAST	I V A T G S E V T P F P G I E I D E E
sp P0A9P0 DLDH_ECOLI	I I A A G S R P I Q L P F I P H E D P
sp P14218 DLDH_PSEFL	I I A S G S R P V E I P P A P L S D D
	→
	βF1 423
sp P09622 DLDH_HUMAN	DRV L G A H I L G P G A G E M V N E A A
sp P09623 DLDH_PIG	DRV L G A H I I G P G A G E M I N E A A
sp P09624 DLDH_YEAST	E R I L G A H I I G P N A G E M I A E A G
sp P0A9P0 DLDH_ECOLI	H R V I G G A I V G T N G G E L L G E I G
sp P14218 DLDH_PSEFL	DRV L G V H I V G P S A A E L V Q Q G A
	→ → → → →
	βG5 α11

Figure 1. Sequence alignments of the Pro-154 and Pro-423 regions of human E3 with the corresponding regions of E3s from various sources (from top to bottom; human, pig, yeast, *Escherichia coli* and *Pseudomonas fluorescens*). The UniProtKB ID is shown and Pro-154, Pro-423 and the corresponding residues are underlined. Alignment analysis was performed using MAFFT program at ExPASy Proteomics Server (Swiss Institute of Bioinformatics).

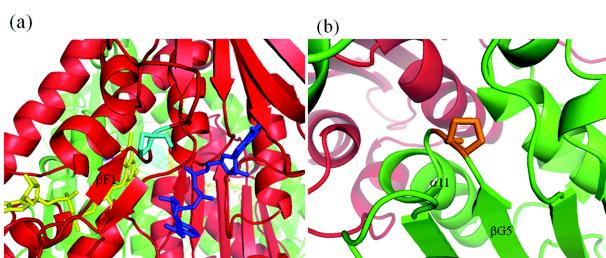


Figure 2. Location of Pro-154 (a) and Pro-423 (b) in human E3. Two subunits of human E3 are shown as cartoons, with each secondary structure portrayed in a single color (red and green, respectively). FAD (yellow), NAD⁺ (blue), Pro-154 (cyan) and Pro-423 (orange) are shown as sticks. Pro-154 is a part of random coil structure between β -sheet F1 and α -helix 4 and is located close to NAD⁺ whereas Pro-423 is a part of β -turn structure between β -sheet G5 and α -helix 11. The structure was drawn using the PyMOL program (DeLano Scientific LLC). The PDB ID for the human E3 structure is 1ZMC.

affected by a Pro-154 to Ala mutation, which affects the random coil structure between β -sheet F1 and α -helix 4? Second, to what extent is the structure and function of human E3 affected by a Pro-423 to Ala mutation, which influences the β -turn structure between β -sheet G5 and α -helix 11?

EXPERIMENTAL SECTION

Materials

The electrophoresis reagents, imidazole, iminodiacetic acid sepharose 6B, lipoamide, and NAD⁺ were obtained from Sigma-Aldrich (St. Louis, USA). Dihydrolipoamide was synthesized by the reduction of lipoamide using sodium borohydride. Isopropyl- β -D-thiogalactopyranoside (IPTG) was purchased from Promega (Madison, USA). *E. coli* XL1-Blue containing a human E3 expression vector pPROEX-1:E3 was a generous gift from Dr. Mulchand S. Patel of University at Buffalo, the State University of New York. The primers and dNTP were procured from Bioneer (Daejeon, Korea). A Muta-Direct™ Site-Directed Mutagenesis Kit was supplied by iNtRON Biotechnology (Seongnam, Korea). The Ni-NTA His-Bind Resin was acquired from QIAGEN (Hilden, Germany).

Site-directed mutagenesis

Site-directed mutagenesis was carried out using a mutagenesis kit with two mutagenic primer pairs, as shown in Table S1. 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

A 3 ml sample of an overnight culture of *E. coli* DH5 α containing the human E3 mutant expression vector was used to inoculate 1 L of LB medium containing ampicillin (100 μ 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 growing temperature was changed to 30 °C and the cells were allowed to grow overnight. The overnight culture was harvested by centrifugation at 4000×g for 5 min. 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 collected by centrifugation at 4000×g for 5 min. The pellets were resuspended in 10 ml of the Binding buffer. The cells were lysed by a sonication treatment and centrifuged at 10,000×g for 20 min.

The supernatant was loaded on to a Ni-NTA His-Bind Resin column. The column had been washed with 2 column volumes of distilled water and then equilibrated with 5 column volumes of the Binding buffer. After loading the supernatant, the column was washed with 10 column volumes of the Binding buffer and then with the same volume of the Binding buffer containing 50 mM imidazole. The E3 mutant was eluted with the Binding buffer containing 250 mM imidazole.

SDS-polyacrylamide electrophoresis

SDS-PAGE analysis of proteins was performed in 12% SDS-PAGE gel. The gel was stained with Coomassie blue after electrophoresis.⁵

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 variable concentrations of the substrates, dihydrolipoamide and NAD⁺, to determine kinetic parameters. The activity was recorded spectrophotometrically by observing the reduction of NAD⁺ at 340 nm with a SPECORD200 spectrophotometer (Analytik Jena AG, Jena, USA). One unit of activity is defined as 1 μ mol of NAD⁺ reduced per min. The data were 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). Samples were excited at 296 nm and the emissions were recorded from 305 nm to 580 nm. The data were transferred to an ASCII file and the spectra were drawn using the MicroCal

Origin program (Photon Technology International, South Brunswick, USA).

RESULTS AND DISCUSSION

The site-directed mutagenesis method is a useful tool for the structure-function study of human E3 and other proteins.⁶ Site-directed mutagenesis was carried out using a mutagenesis kit. Two mutagenic primers were used for the mutations. The mutant was expressed in *E. coli* by IPTG induction (1 mM). Purification of the mutants was performed using a nickel affinity column. The purification steps were followed by SDS-polyacrylamide gel electrophoresis (Fig. S1). The gel showed that the mutants were highly purified.

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. The data were analyzed using a SigmaPlot Enzyme Kinetics Module. The program generated double reciprocal plots (Fig. S2). The plots revealed parallel lines, indicating that both mutants also catalyze the reaction through a Ping Pong Bi Bi mechanism. The program also provides kinetic parameters directly without the need for secondary plots. Table 1 lists the kinetic parameters of the mutants. The k_{cat} value of the P154A mutant increased by 45%, indicating that the mutation improves the catalytic processes of the conversion of substrates to products. The K_m value toward dihydrolipoamide increased by 40%, suggesting that the mutation makes the enzyme binding to dihydrolipoamide less efficient. The K_m value toward NAD⁺ also increased by 40%, suggesting that the mutation makes the enzyme binding to NAD⁺ significantly less efficient. The k_{cat} value of the P423A mutant was reduced by 30%, indicating that the mutation deteriorates the catalytic processes of the conversion of substrates to products. The K_m value toward dihydrolipoamide increased by 10%, indicating that the mutation makes enzyme binding to dihydrolipoamide less efficient. The K_m value toward NAD⁺ increased by 110%, indicating that the mutation makes enzyme binding to NAD⁺ significantly less effective. The

NAD⁺ concentration in cells was determined to be 0.37 mM.^{7,8} Therefore, this significantly inefficient enzyme binding of the mutants to NAD⁺ could be more detrimental inside the cells due to the low cellular concentration of NAD⁺.

Pro-154 is a component of a random coil structure between β-sheet F1 and α-helix 4 and is located close to NAD⁺, whereas Pro-423 is a part of the β-turn structure between β-sheet G5 and α-helix 11 (Fig. 2). The Chou and Fasman secondary structure prediction method predicts that the mutation of Pro-453 to Ala can also affect the β-turn structure.⁹ The changes in the β-turn structure will cause significant structural changes in this region. The amino acid volume of Pro was 112.7 Å³, whereas that of Ala was 88.6 Å³.¹⁰ A Pro to Ala mutation will result in a vacancy with a volume of 24.1 Å³ at residues-154 and 423, which will also remove the conformational rigidity of Pro at the mutation site. This vacancy and conformation freedom will cause structural changes at the mutation sites, which induce alterations in the kinetic parameters of the enzyme. The dihydrolipoamide binding site is located at the *si*-face of FAD, whereas the NAD⁺ binding site is at the *re*-face. The structural changes are not limited to the local structure of the mutation site but are also expanded to other regions of the mutants so that the both apparent mutant binding affinities to dihydrolipoamide and NAD⁺ are simultaneously deteriorated.

Fluorescence spectroscopy was performed to examine any structural changes occurring in the mutants. When enzymes were excited at 296 nm, two fluorescence emissions were observed for both the mutant and wild-type E3s, as shown in Fig. 3. The first emission from 305 nm to 400 nm was due mainly to Trp. The second emission from 480 nm to over 550 nm was due to FAD. In human E3, the Trp fluorescence was quenched due to fluorescence resonance energy transfer (FRET) from Trp to FAD. When the fluorescence spectra were compared, a large difference was found in the 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 P154A mutant (dashed line) was much lower than that (5.2) of the wild-type enzyme (dotted line). This suggests that the FRET from Trp to FAD was disturbed severely in the mutant. The structural changes due to a Pro-154 to

Table 1. Steady state kinetic parameters of 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 ± S.D. of three independent determinations

Human E3s	k_{cat} (s ⁻¹)	K_m toward dihydrolipoamide (mM)	K_m toward NAD ⁺ (mM)
wild-type	899±114	0.64±0.06	0.19±0.02
P154A mutant	1309±20	0.87±0.03	0.27±0.01
P423A mutant	711±17	0.70±0.19	0.40±0.01

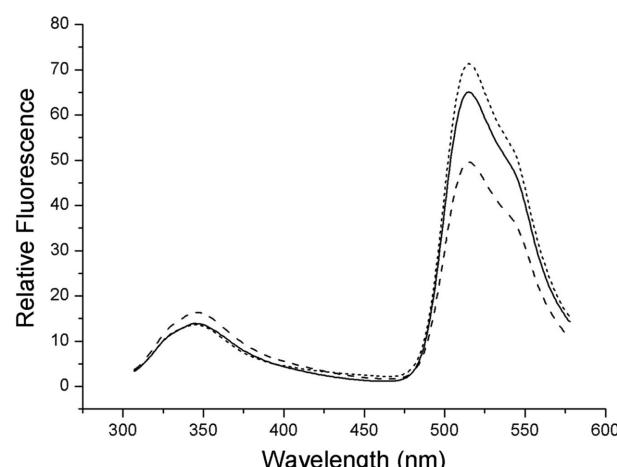


Figure 3. Fluorescence spectra of the P154A mutant (dashed line), P423A mutant (solid line) and wild-type (dotted line) human E3s. Enzymes were excited at 296 nm and the emissions were observed from 305 nm to 575 nm. The data were transferred to an ASCII file and the spectra were then drawn using the MicroCal Origin program.

Ala mutation might have affected the structure of human E3, interfering with energy transfer from the Trp residues to FAD. The ratio (4.7) between the relative intensities of the first and second fluorescence emissions of the P423A mutant (solid line) was also smaller than that of the wild-type enzyme (dotted line). This indicates that the FRET from Trp to FAD was also disturbed in the P423A mutant. The small structural changes due to a Pro-423 to Ala mutation could have affected the structure of human E3, interfering with the FRET from the Trp residues to FAD. The precise structural changes due to the mutation can be revealed only by an X-ray crystallographic study.

This study examined the effects of the Pro-154 to Ala and Pro-423 to Ala mutations on the human E3 structure and function using site-directed mutagenesis, E3 activity measurements, and fluorescence spectroscopy. The Pro-154 to Ala mutation in human E3 makes enzyme binding to both dihydrolipoamide and NAD⁺ inefficient and causes structural changes, which interfere with the efficient FRET

from Trp to FAD in the enzyme. The Pro-423 to Ala mutation in human E3 also makes mutant binding to both dihydrolipoamide and NAD⁺ less efficient and causes structural changes that interfere with the efficient FRET from the Trp residues to FAD. These findings suggest that the conservation of both Pro-154 and Pro-423 in human E3 is important for the efficient enzyme binding to both dihydrolipoamide and NAD⁺ and for efficient FRET from Trp to FAD.

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Supporting Information. Additional supporting information is available in the online version of this article.

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