Journal of the Korean Chemical Society
2016, *Vol. 60, No. 1*
Printed in the Republic of Korea
http://dx.doi.org/10.5012/jkcs.2016.60.1.88

단신 (Notes)

Characterization of a Naturally Occurring Mutation (Arg-447 to Gly) in Human Dihydrolipoamide Dehydrogenase of Patients with Neurological Deterioration

Hakjung Kim
Department of Chemistry, College of Natural Science, Daegu University, Gyoungsan 38453, Korea.
E-mail: hjkim@daegu.ac.kr
(Received September 7, 2015; Accepted November 19, 2015)

Key words: Dihydrolipoamide dehydrogenase, Pyridine nucleotide-disulfide oxidoreductase, Flavoenzyme, Dihydrolipoamide deficiency, α-Keto acid dehydrogenase complexes

Dihydrolipoamide dehydrogenase (E3) (dihydrolipoamide:NAD^+ oxidoreductase; EC 1.8.1.4) is an integral component in pyruvate,[1] α-ketoglutarate,[1] branched-chain α-keto acid dehydrogenase complexes,[1] and the glycine cleavage system.[2] E3 facilitates reoxidation of the dihydrolipoyl group of the acyltransferase components of α-keto acid dehydrogenase complexes and the hydrogen-carrier protein of the glycine cleavage system. E3 is a homodimeric flavoenzyme containing a single FAD at each subunit (*Figure* 1).

E3 is a member of the pyridine nucleotide-disulfide oxidoreductase family along with glutathione reductase, thioredoxin reductase, mercuric reductase, and trypanothione reductase.[3] Their structures are similar, having four structural domains (FAD, NAD, central and interface domains). Their homodimeric structures contain an active disulfide center and a FAD domain in each subunit. Their catalytic mechanisms are also similar, catalyzing electron transfer between the pyridine nucleotides (NAD^+ or NADPH) and their specific substrates through FAD and the active disulfide center.

Patients with an E3 deficiency present with a variety of clinical symptoms, ranging from Leigh syndrome, serious neurological disease, to exertional fatigue.[4] Because E3 is a common essential component in three α-keto acid dehydrogenase complexes, a reduction of E3 activity leads to a decrease in the activities of all three complexes. Patients show increased urinary excretion of keto acids, and elevated blood levels of lactate, pyruvate and branched chain amino acids. The central nervous system, such as the brain is most commonly affected by these genetic defects because the system normally uses glucose as a principle energy source. One mutation found in patients with low E3 activity is the Arg-447 to Gly mutation.[5] Patients with this mutation suffer from neurological deterioration and generally die within 3 years.

Figure 2 presents the sequence alignment of the sequence around the Arg-447 region of human E3 with the corresponding regions of E3s from a range of sources, such as pigs, yeast, *Escherichia coli*, and *Pseudomonas fluorescens*. Arg-447 is conserved in eukaryotic E3s, suggesting that Arg-447 might be important for the function and structure of these E3s, including human E3. Arg-447 is located immediately behind short α-helix structure 12 (*Figure* 3), which is composed of 5 amino acids and a component of the interface domain. This study examined the effects of the naturally occurring Arg-447 to Gly mutation on the human E3 structure and function, leading to neurological deterioration.

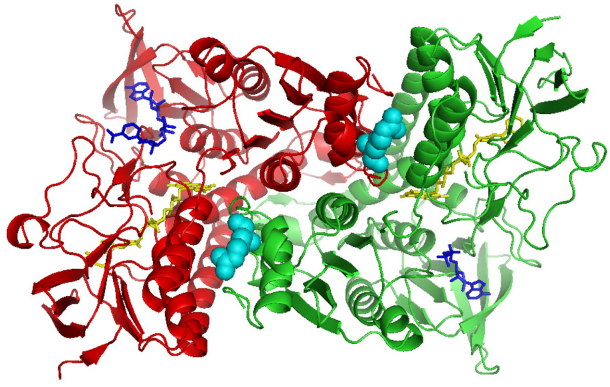

***Figure* 1.** Homodimeric structure of human E3. Two subunits are shown as cartoons, representing the secondary structures in a single color (red and green, respectively). FAD (yellow), NAD^+ (blue) and Arg-447 (cyan) are shown as sticks and spheres, respectively. The structure was drawn using the PyMOL program (DeLano Scientific LLC). The PDB ID for the human E3 structure is 1ZMC.[14]

E3s	Amino Acid Sequences 447
sp|P09622|DLDH_HUMAN	EYGASCEDIARVCHAHPTLSE
sp|P09623|DLDH_PIG	EYGASCEDIARVCHAHPTLSE
sp|P09624|DLDH_YEAST	EYGASAEDVARVCHAHPTLSE
sp|P0A9P0|DLDH_ECOLI	EMGCDAEDIALTIHAHPTLHE
sp|P14218|DLDH_PSEFL	EFGTSAEDLGMMVFSHPTLSE

	α12

***Figure* 2.** Sequence alignments of the Arg-447 region of human E3 with the corresponding regions of E3s from a range of sources (from top to bottom; human, pigs, yeast, *Escherichia coli*, and *Pseudomonas fluorescens*). The UniProtKB IDs and amino acid sequences from residue-437 to residue-457 are shown. Arg-447 and the corresponding residues are underlined. Alignment analysis was performed using the MAFFT program on a ExPASy Proteomics Server (Swiss Institute of Bioinformatics).

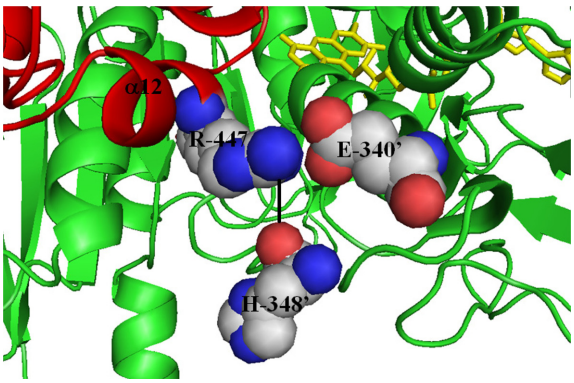

***Figure* 3.** Location of Arg-447 in human E3. Two subunits are shown as cartoons, representing the secondary structures in a single color (red and green, respectively). FAD (yellow) is shown as sticks. Arg-447, Glu-340' and His-348' are shown as spheres (the blue, red and white color represents nitrogen, oxygen and carbon, respectively). Arg-447 forms an ionic interaction with Glu-340' and a hydrogen bond with the backbone CO group of His-348' from the other subunit (solid line). The structure was drawn using the PyMOL program (DeLano Scientific LLC). The PDB ID for the human E3 structure is 1ZMC.[14]

EXPERIMENTAL SECTION

Materials

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 electrophoresis reagents, imidazole, iminodiacetic acid sepharose 6B, lipoamide, and NAD^+ were purchased from Sigma-Aldrich (St. Louis, USA). Dihydrolipoamide was obtained by the reduction of lipoamide using sodium borohydride. Isopropyl-β-D-thiogalactopyranoside (IPTG) was purchased from Promega (Madison, USA). Ni-NTA His-Bind Resin was obtained from QIAGEN (Hilden, Germany). The primers and dNTP were purchased from Bioneer (Daejeon, Korea). A Muta-Direct™ Site-Directed Mutagenesis kit was purchased from iNtRON Biotechnology (Seongnam, Korea).

Site-directed mutagenesis

Site-directed mutagenesis was performed using a mutagenesis kit (iNtRON Biotechnology, Sungnam, Korea). Two mutagenic primers were used for the mutation. Primer A (5'-GTGAAGATATAGCTGGAGTCTGTCATGCACATC-3': the mismatched bases are underlined) is a sense oligomer with a point mutation to convert Arg-447 (AGA) to Gly (GGA). Primer B (5'-GATGTGCATGACAGACTC-CAGCTATATCTTCAC-3': the mismatched bases are underlined) is the corresponding anti-sense oligomer of primer A. PCR was carried out using the human E3 expression vector, pPROEX-1:E3, as a template in a programmable PCR machine. The entire DNA sequence of the human E3 coding region was sequenced to confirm the integrity of the DNA sequences other than the anticipated mutation.

Expression and purification of the human E3 mutant

Three mL of an overnight culture of *Escherichia coli* XL1-Blue, possessing the human E3 mutant expression vector, were 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, which was followed by the addition of IPTG to a final concentration of 1 mM. The growth 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. The cell pellets were resuspended 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 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, which had been washed with 2 column volumes of distilled water and 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 a binding buffer containing 250 mM 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 E3 activity was assayed at 37 ºC 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 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 min. The kinetic parameters were determined 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 structure-function studies of human E3 and other proteins.[7–9] Arg-447 was mutated site-specifically to Gly by site-directed mutagenesis to examine the effects of an Arg-447 to Gly mutation on the human E3 structure and function, leading to neurological deterioration,. The site-directed mutagenesis with the mutagenic primers resulted in the construction of the mutant E3 expression vectors. The mutants were expressed in *Escherichia coli* and purified by a Ni-NTA His-Bind Resin column. The SDS-PAGE gel revealed the mutants to be highly purified (*Figure* 4).

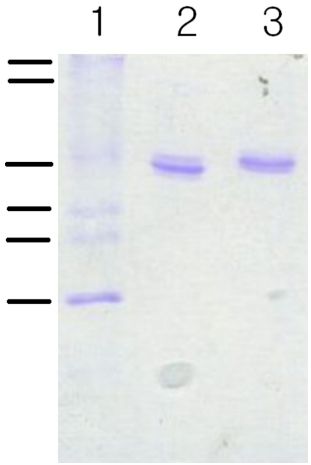

***Figure* 4.** SDS-PAGE gel (12%) for the purified mutant E3. Lane 1, molecular weight markers (from top to bottom; myosin 200.0 kDa, β-galactosidase 116.0 kDa, glutamic dehydrogenase 55.0 kDa, ovalalbumin 45.0 kDa, glyceraldehyde-3-phosphate dehydrogenase 36.0 kDa, carbonic anhydrase 29.0 kDa); lane 2, the previously purified recombinant human E3 as a control; lane 3 the purified mutant E3.

To determine the kinetic parameters, an E3 assay was performed at 37 ºC 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 kinetic experiments were carried out in triplicate. The data was analyzed using a SigmaPlot Enzyme Kinetics Mod-

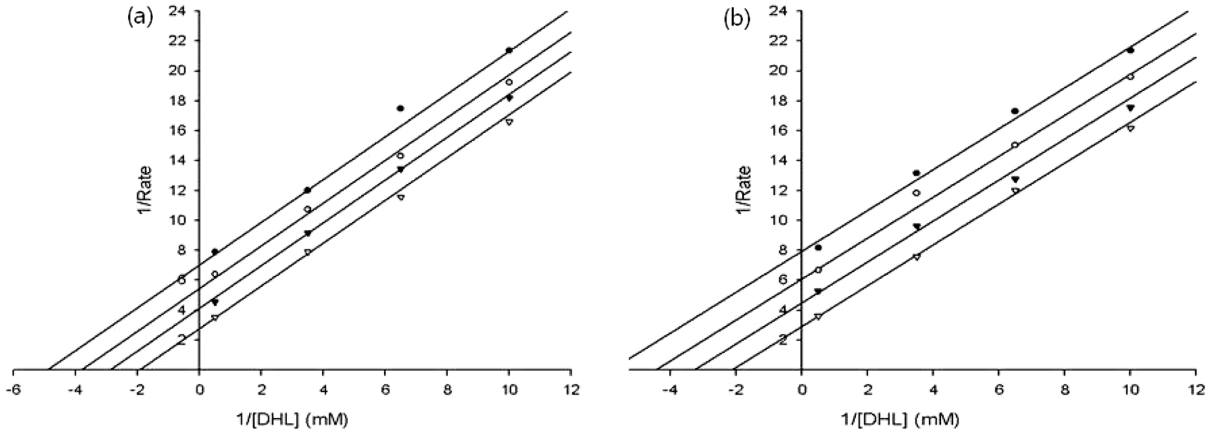

***Figure* 5.** Double reciprocal plots for the normal (a) and mutant (b) human E3s. E3 activities were determined at 37 ºC in a 50 mM potassium phosphate buffer (pH 8.0) containing 1.5 mM EDTA with variable concentrations of the substrates, dihydrolipoamide (DHL) and NAD^+. The plots were drawn with the SigmaPlot Enzyme Kinetics Module program. The NAD^+ concentrations from 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.

***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. Values are mean ± S.D. 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 dl[a] (s^{-1}/mM)	k_{cat}/K_m toward NAD^+ (s^{-1}/mM)
Normal	899±114	0.64±0.06	0.19±0.02	1405	4732
R447G mutant	739±51	0.55±0.04	0.24±0.04	1344	3079

[a]Dihydrolipoamide.

ule (Systat Software Inc., San Jose, USA). The program generated double reciprocal plots (*Figure* 5) that showed parallel lines, indicating that the mutant also catalyzes the reaction *via* a ping pong mechanism of the normal enzyme.[10] 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 mutant was reduced by 18%, suggesting that the mutation deteriorates the catalytic conversion step of the substrates to products. The K_m value toward dihydrolipoamide decreased by 14%, indicating that the mutation improved the efficiency of enzyme binding to dihydrolipoamide. On the other hand, the K_m value toward NAD^+ increased by 26%, suggesting that the mutation reduces significantly the efficiency of enzyme binding to NAD^+. The catalytic efficiency (k_{cat}/K_m) toward dihydrolipoamide decreased by 4.3% because a lower K_m value toward dihydrolipoamide compensates for the reduced k_{cat} value. On the other hand, the catalytic efficiency toward NAD^+ decreased by 35%, suggesting that the mutation makes the enzyme significantly less efficient toward NAD^+. These significantly reduced apparent binding affinity and catalytic efficiency of the mutant toward NAD^+ could be more detrimental inside the cells because of the low cellular NAD^+ concentration (0.37 mM).[11,12] The K_m value of the mutant toward NAD^+ is 0.24 mM. This means that the mutant needs at least 0.48 mM of cellular NAD^+ concentration for its maximum apparent binding affinity toward NAD^+. The maximum apparent binding affinity toward NAD^+ of the mutant is deteriorated by 23% due to the increased K_m value of the mutant toward NAD^+ and the low cellular NAD^+ concentration.

Fluorescence spectroscopy was performed to examine the structural changes in the mutant. Two fluorescence emissions were observed for both the mutant and normal E3s after exciting the enzymes at 296 nm (*Figure* 6). The first emission from 305 nm to 400 nm was attributed mainly to Trp. The second emission from 480 nm to >550 nm was assigned 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

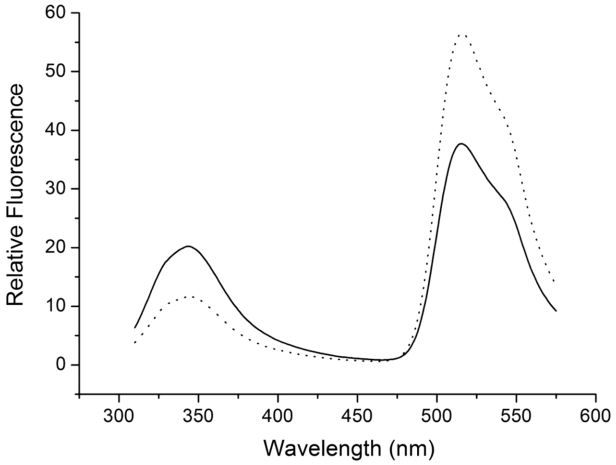

***Figure* 6.** Fluorescence spectra of the mutant (solid line) and normal (dotted line) human E3s. Enzymes were excited at 296 nm and the emissions were observed from 305 nm to 575 nm. The data were transferred to an ASCII file and the spectra were drawn using the MicroCal Origin program.

of the E3s were compared, a noticeable difference in the ratio between the relative intensities of the first and second fluorescence emissions was observed. The ratio (1.9) between the relative intensities of the first and second fluorescence emissions of the mutant (solid line) was significantly lower than that (4.9) of the normal enzyme (dotted line). This suggests that FRET from Trp to FAD had been disturbed in the mutant. Moreover, the structural changes that occurred in the human E3 mutant interfered with FRET from Trp to FAD. The positively charged side chain of Arg-447 forms an ionic interaction with the negatively charged side chain of Glu-340' and a hydrogen bond with the backbone the CO group of His-348' from the other subunit (*Figure* 3). The Arg-447 to Gly mutation, having just one hydrogen atom as its side chain, will eliminate all these interactions. The mutation also results in a considerable vacancy of 113.3 $Å^3$ in the region because the amino acid volume of Arg is 173.4 $Å^3$, whereas that of Gly is 60.1 $Å^3$.[13] These factors will cause structural changes in the mutant that will alter the kinetic parameters of the mutant. The precise structural changes can only be revealed by an X-ray crystallographic study.

The effects of a naturally occurring mutation (Arg-447 to Gly) in human E3 on the structure and function of the enzyme were examined at the enzyme level by site-directed mutagenesis, E3 activity measurements and fluorescence spectroscopy. An Arg-447 to Gly mutation results in structural changes that alter the fluorescence spectrum of human E3 and interfere with efficient FRET from Trp to FAD. The structural changes affect the kinetic parameters of the mutant. In particular, the apparent binding affinity and catalytic efficiency of the mutant toward NAD^+ deteriorated significantly, suggesting that the mutation makes the enzyme significantly less efficient toward NAD^+. This reduced catalytic function of the mutant toward NAD^+ could be more detrimental due to the limited availability of cellular NAD^+. In conclusion, the conservation of Arg-447 in human E3 is important for its proper structure and function.

Acknowledgements. The author thanks Dr. Mulchand S. Patel (University at Buffalo, the State University of New York) for a generous gift of an *E. coli* XL1-Blue containing a human E3 expression vector. The author also thanks to Dongseok Lee for technical assistance. This research was supported in part by the Daegu University Research Grant.

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