Journal of the Korean Chemical Society
2019, *Vol. 63, No. 2*
Printed in the Republic of Korea
https://doi.org/10.5012/jkcs.2019.63.2.134

Note

Characterization of Human Dihydrolipoamide Dehydrogenase Mutant Showing Significantly Decreased Catalytic Efficiency

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(Received November 12, 2018; Accepted December 24, 2018)

Key words: Dihydrolipoamide dehydrogenase, Pyridine nucleotide-disulfide oxidoreductase, Flavoenzyme

Dihydrolipoamide dehydrogenase (E3) (dihydrolipoamide:NAD^+ oxidoreductase; EC 1.8.1.4) is an essential component in the pyruvate dehydrogenase complex as well as in α-ketoglutarate and branched-chain α-keto acid dehydrogenase complexes[1] and the glycine cleavage system.[2] E3 facilitates reoxidation of the dihydrolipoyl prosthetic group of the acyltransferase components of the dehydrogenase complexes and the hydrogen-carrier protein of the glycine cleavage system. A decrease in E3 activity can affect the activities of all three complexes because E3 is commonly present in these complexes. This results in increased urinary excretion of α-keto acids, elevated blood lactate, pyruvate, and branched chain amino acids. Patients with an E3 deficiency die young because it is a fatal genetic defect that affects the central nervous system, such as the brain, which leads to serious diseases including Leigh syndrome with permanent lactic acidaemia and mental retardation.[3]

E3 is a homodimeric flavoenzyme that contains one FAD as a prosthetic group at each subunit (*Fig.* 1).[4] The subunit consists of 474 amino acids with a molecular mass of 50,216 daltons.[5] E3 belongs to the pyridine nucleotide-disulfide oxidoreductase family along with glutathione reductase, thioredoxin reductase, mercuric reductase, and trypanothione reductase.[6] Their catalytic mechanisms are similar. All have homodimeric structures containing an active disulfide center and a FAD in their each subunit. Through the FAD and active disulfide center, they catalyze electron transfer between the pyridine nucleotides (NAD^+ or NADPH) and their specific substrates. These enzymes consist of four structural domains (FAD, NAD, central and interface domains).[7]

Knowledge of the binding modes of FAD and NAD^+ to human E3 can be predicted from the X-ray crystallographic structure of human E3.[4] On the other hand, the binding mode of dihydrolipoamide has not been revealed because the human E3 structure with dihydrolipoamide has not been determined. Leu-99 is located at the presumed dihydrolipoamide binding channel (*Fig.* 1), suggesting that it might be involved in enzyme binding to dihydrolipoamide. Leu-99 is located at the middle of the longest a-helix 3 in human E3 (*Fig.* 2), which is composed of 26 amino acids and a component of the FAD domain.

Fig. 3 presents the sequence alignments of the sequence around Leu-99 of human E3 with the corresponding sequences from various E3s, such as pigs, yeast, *Escherichia coli* and *Pseudomonas fluorescens*. Leu-99 is absolutely conserved in the E3s, suggesting that it might be important for the structure and function of E3, including human E3.

The purpose of this research is to examine the suggestion that Leu-99 might be involved in in enzyme binding to dihydrolipoamide and to assess the importance of Leu-99 in the human E3 function. Leu-99 was mutated site-specifically to Ala to achieve this purpose. The effects of this arti-

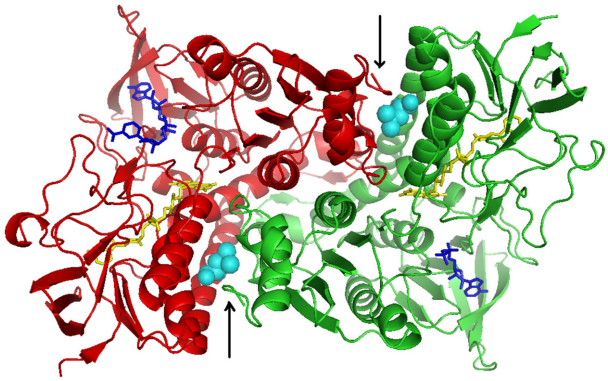

***Figure* 1.** Homodimeric structure of human E3. Two subunits are shown as cartoons, representing the secondary structures in a single color (red and green, respectively). FAD (yellow), NAD^+ (blue), and Leu-99 (cyan) are shown as sticks and spheres, respectively. The arrows indicate the dihydrolipoamide binding channels. The structure was drawn using the PyMOL program (DeLano Scientific LLC). The PDB ID for the human E3 structure is 1ZMC.

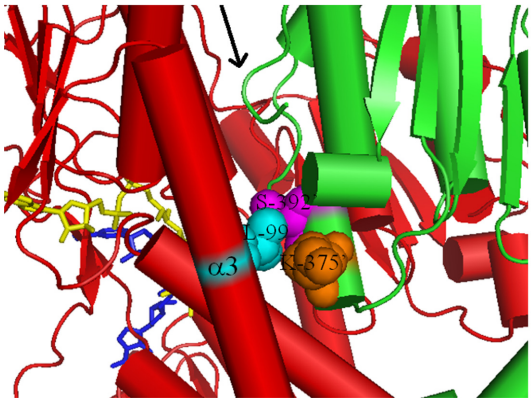

***Figure* 2.** Location of Leu-99 in human E3. Two subunits of human E3 are shown as cartoons, representing secondary structures in a single color (red and green, respectively). FAD (yellow), NAD^+ (blue), Ser-392' (magenta), Lys-375' (orange), and Leu-99 (cyan) are shown as sticks and spheres, respectively. Leu-99 is a part of the dihydrolipoamide binding channel as indicated by an arrow and makes van der Waals interactions with Ser-392' and Lys-395' from the other subunit. The structure was drawn using the PyMOL program (DeLano Scientific LLC). The PDB ID for the human E3 structure is 1ZMC.

E3	Amino acid sequence
	99
sp|P09622|DLDH_HUMAN	DKMMEQKSTAVKALTGGIAHLFKQN
sp|P09623|DLDH_PIG	EKMMEQKSNAVKALTGGIAHLFKQN
sp|P09624|DLDH_YEAST	ANFQKAKDDAVKQLTGGIELLFKKN
sp|P0A9P0|DLDH_ECOLI	DKIRTWKEKVINQLTGGLAGMAKGR
sp|P14218|DLDH_PSEFL	PAMVARKANIVKNLTGGIATLFKAN

	α3

***Figure* 3.** Sequence alignment of the Leu-99 region of human E3 with the corresponding regions of E3s from a range of sources (from top to bottom; human, pig, yeast, *Escherichia coli* and *Pseudomonas fluorescens*). The UniProtKB ID and amino acid sequence from residue-86 to residue-110 are shown. Leu-99 and the corresponding residues are underlined. Alignment analysis was performed using the MAFFT program on the ExPASy Proteomics Server (Swiss Institute of Bioinformatics).

ficial mutation on the human E3 function were examined by measuring the E3 activity.

EXPERIMENTAL SECTION

Materials

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). The *Escherichia 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 and the State University of New York. 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 obtained from QIAGEN (Hilden, Germany).

Site-directed Mutagenesis

Two mutagenic primers were used for the Leu-99 to Ala mutation. Primer A (5'-CTGCAGTAAAAGCTGCAACAGGTGGAATTGCCC-3': the mismatched bases are underlined) is an anti-sense oligomer with point mutations to convert Leu-99 (TTA) to Ala (GCA). Primer B (5'-GGGCAATTCCACCTGTTGCAGCTTTTACTGCAG-3': the mismatched bases are underlined) is the corresponding 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 mutations.

Expression and Purification of the Human E3 Mutant

A 3 ml sample of an overnight culture of *Escherichia coli* DH5a containing the human E3 mutant expression vector was used to inoculate 1 L of LB medium containing ampicillin (100mg/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 × g for 5 min. The cell pellets were washed with potassium phosphate buffer (50 mM, pH 8.0) containing 100 mM NaCl and 20 mM imidazole (Binding buffer), and 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, 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.

The purified enzyme concentration was determined by

measuring the absorbance at 458 nm, which indicated the prosthetic group FAD content of the enzyme. The molar absorption coefficient of the prosthetic group FAD of human E3 is 11.3×10^3 M^{-1} cm^{-1}.

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.[8]

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, dihydrolipoamide, and NAD^+, to determine the kinetic parameters. The activity was measured 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).

RESULTS AND DISCUSSION

To examine the importance of Leu-99 in the human E3 structure and function, Leu-99 was mutated site-specifically to Ala. The mutant was expressed in *Escherichia coli* and purified using 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 were analyzed using the SigmaPlot Enzyme Kinetics Module (Systat Software Inc., San Jose, USA). The double reciprocal plots revealed 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* 1 lists the kinetic parameters of the mutant and wild-type human E3s. The k_{cat} value of the mutant decreased 56-fold, indicating that the mutation deteriorates the catalytic power of the enzyme substantially. The K_m value toward dihydrolipoamide was reduced by 48%, indicating that the mutation makes the enzyme more efficient to dihydrolipoamide. This indicates that Leu-99 is involved in enzyme binding to dihydrolipoamide, which is consistent with the location of Leu-99, the dihydrolipoamide binding channel. In contrast, the K_m value toward NAD^+ was increased 6.8-fold, indicating that the mutation makes the enzyme significantly less efficient to NAD^+. The catalytic efficiency (k_{cat}/K_m) of the mutant toward dihydrolipoamide was decreased 27-fold, indicating that the mutant becomes a significantly less efficient enzyme toward dihydrolipoamide. The catalytic efficiency of the mutant toward NAD^+ was reduced 394-fold, indicating that the mutation makes the enzyme substantially less efficient toward NAD^+. The NAD^+ concentration in the cells was determined to be 0.37 mM.[9,10] The substantially higher K_m value and lower catalytic efficiency toward NAD^+ could be more detrimental inside the cells because of the low cellular NAD^+ concentration. A Leu-99 to Ala mutation could cause structural changes, resulting in altered kinetic parameters of the mutant. Leu-99 can form van der Waals interactions with Ser-392' and Lys-395'

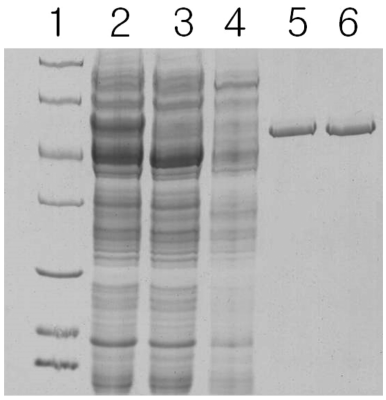

***Figure* 4.** SDS-polyacrylamide gel for the purification of the mutant E3. Lane 1, molecular weight markers (from top to bottom, β-galactosidase 116.3 kDa, bovine serum albumin 66.2 kDa, ovalalbumin 45.0 kDa, lactate dehydrogenase 35.0 kDa, REase Bsp981 25 kDa, β-lactoglobulin 18.4 kDa, lysozyme 14.4 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* 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 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)
Wild-type[b]	899±114	0.64±0.06	0.19±0.02	1405	4732
L99A mutant	16.2±0.1	0.31±0.00	1.30±0.00	52	12

[a]Dihydrolipoamide.
[b]From the reference 12.

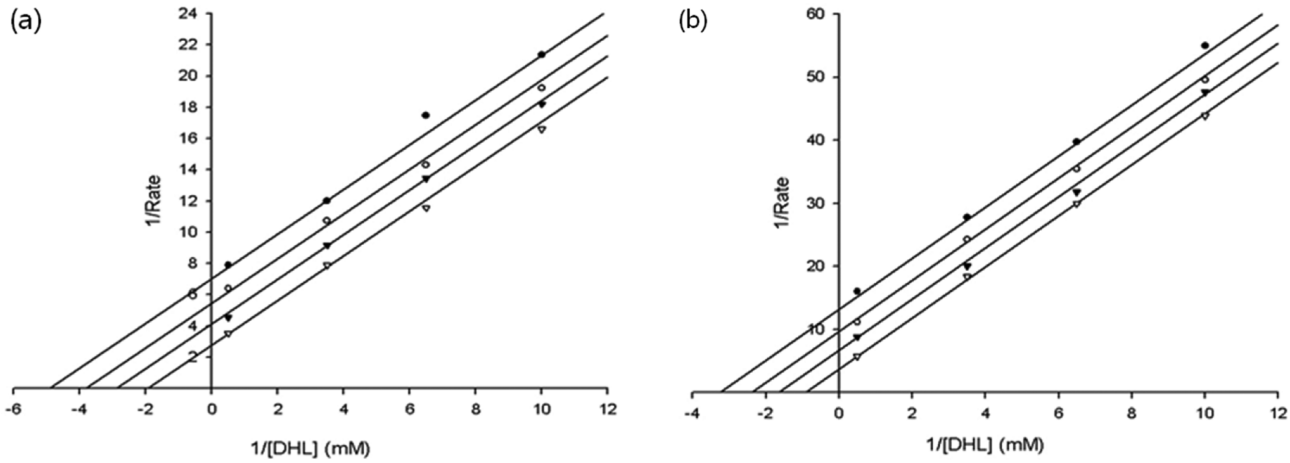

***Figure* 5.** Double reciprocal plots for the wild-type (a) and mutant (b) human E3s. The E3 activities were determined at 37 °C in a 50 mM potassium phosphate buffer (pH 8.0) containing 1.5 mM EDTA with variable concentrations of the substrates, dihydrolipoamide (DHL) and NAD^+. Plots were drawn using 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.

from the other subunit (*Fig.* 2). A mutation of Leu-99 to Ala will affect these interactions. The amino acid volume of Leu is 166.2 $Å^3$, whereas that of Ala is 88.6 $Å^3$.[11] The mutation will give a large vacancy of 77.6 $Å^3$ at residue-99. These alterations could cause structural changes in this region, which could alter the kinetic parameters of the mutant.

In this study, the effects of the site-specific mutation of Leu-99 to Ala in human E3 on the structure and function of the enzyme were examined using site-directed mutagenesis, E3 activity measurements. A mutation of Leu-99 to Ala in human E3 deteriorates the catalytic power of the enzyme significantly, indicating that Leu-99 is very important for the proper catalytic function of human E3. The mutant shows contrary K_m values toward the substrates, a smaller K_m value toward dihydrolipoamide but a larger K_m value toward NAD^+, indicating that the mutation gives the enzyme different apparent binding affinities toward these substrates. These findings suggest that the conservation of Leu-99 in human E3 is very important for the proper catalytic function of the enzyme.

Acknowledgments. 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. This research was supported in part by the Daegu University Research Grant.

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