Journal of the Korean Chemical Society
2020, *Vol. 64, No. 3*
Printed in the Republic of Korea
https://doi.org/10.5012/jkcs.2020.64.3.141

Communication

Characterization of a Human Dihydrolipoamide Dehydrogenase Mutant Showing Significantly Decreased Catalytic Efficiency toward NAD^+

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(Received February 8, 2020; Accepted March 14, 2020)

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

Dihydrolipoamide dehydrogenase (E3) (dihydrolipoamide: NAD^+ oxidoreductase; EC 1.8.1.4) is a flavin-dependent homodimeric enzyme containing one FAD as a prosthetic group at each subunit (*Fig.* 1).[1] Each subunit of human E3 is composed of 474 amino acids with a molecular mass of 50,216 daltons.[2] Three a-keto acid dehydrogenase complexes (pyruvate, a-ketoglutarate and branched-chain a-keto acid dehydrogenase complexes) have E3 as a common component.[3] E3 catalyzes the reoxidation of the dihydrolipoyl prosthetic group attached to the lysyl residue(s) of the acyltransferase components of the three a-keto acid dehydrogenase complexes. Because E3 is an essential component in three a-keto acid dehydrogenase, the decrease of E3 activity can affect the activities of all three complexes. This results in increased urinary excretion of a-keto acids, elevated blood lactate, pyruvate, and branched chain amino acids. Patients with an E3 deficiency normally die young because such a deficiency is a detrimental genetic defect that harms the central nervous system. Leigh syndrome with recurrent episodes of hypoglycemia and ataxia, permanent lactic acidaemia and mental retardation can be manifested.[4]

Pro is an imino acid with an exceptional conformational rigidity. Pro usually plays very important roles in the protein structure such as a turn. *Fig.* 2 shows a sequence alignment of the Pro-355 region of human E3 with the corresponding regions of E3s from various sources such as pig, yeast, *Escherichia coli* and *Pseudomonas fluorescens*. Pro-355 is absolutely conserved in the E3s, suggesting that it might be important for the structure and function of most E3s including human E3. Pro-355 is located at the end of a random coil structure between the long a-helix structure 8, consisting of 16 amino acids, and the very short b-sheet structure G1, composed of 3 amino acids. These structures are located at the boundary region between the central and interface domains. Pro-355 can form van der Waals interactions with His-329, which is a component of the a-helix structure 8 and important for the structure and function of human E3 (*Fig.* 3).[5]

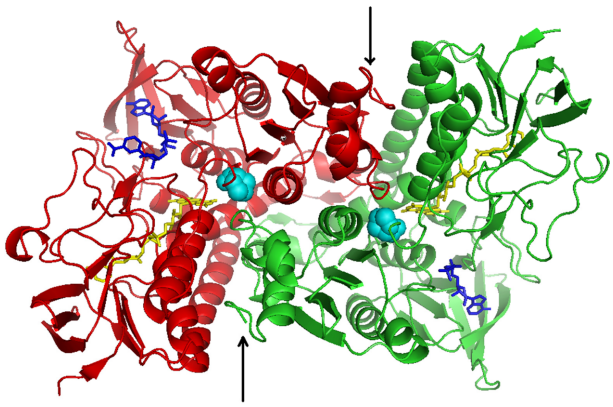

***Figure* 1.** Homodimeric structure of human E3. Two subunits are shown as cartoons, representing secondary structures in a single color (red and green, respectively). FAD (yellow), NAD^+ (blue) and Pro-355 (cyan) are shown as sticks and spheres, respectively. The structure was drawn using the PyMOL program (DeLano Scientific LLC). The PDB ID for the human E3 structure is 1ZMC.

E3	Amino acid sequence
	355
sp|P09622|DLDH_HUMAN	AVHIDYNCVPSVIYTHPEV
sp|P09623|DLDH_PIG	AVHIDYNCVPSVIYTHPEV
sp|P09624|DLDH_YEAST	HGHVNYNNIPSVMYSHPEV
sp|P0A9P0|DLDH_ECOLI	KHYFDPKVIPSIAYTEPEV
sp|P14218|DLDH_PSEFL	KAQMNYDLIPSVIYTHPEI
	-->
	βG1

***Figure* 2.** Sequence alignment of the Pro-355 region 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-355, Pro-362 and the corresponding residues are underlined. Alignment analysis was performed using MAFFT program at ExPASy Proteomics Server (Swiss Institute of Bioinformatics).

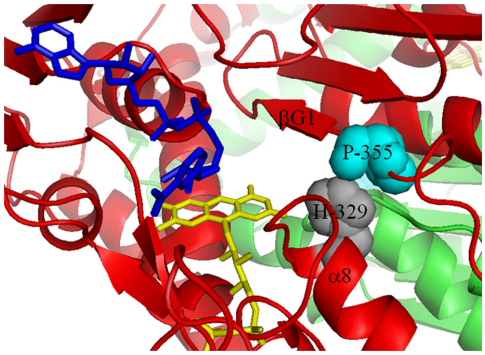

***Figure* 3.** Location of Pro-355 in human E3. Two subunits of human E3 are shown as cartoons, representing secondary structures in a single color (red and green, respectively). FAD (yellow), NAD^+ (blue), His-329 (grey) and Pro-355 (cyan) are shown as sticks and spheres, respectively. Pro-355 is the end of a random coil structure between α-helix 8 and β-sheet G1. Pro-355 is located close to His-329 within a van der Waals distance. The structure was drawn using the PyMOL program (DeLano Scientific LLC). The PDB ID for the human E3 structure is 1ZMC.

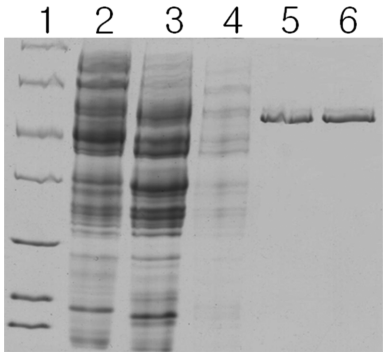

***Figure* 4.** SDS-polyacrylamide gel for the purification of the mutant. Lane 1, molecular weight markers (from top to bottom, β-galactosidase 116.3 kDa, bovine serum albumin 66.2 kDa, ovalalbumin 45.0 kDa, lactate dehydrogenase 35.0 kDa, REase Bsp981 25 kDa, β-lactoglobulin 18.4 kDa, lysozyme 14.4 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.

To examine the importance of Pro-355 in human E3 on the structure and function of the enzyme, Pro-355 was mutated site-specifically to Ala using site-directed mutagenesis. Two mutagenic primers were used for the mutations. Primer A (5'-CTACAATTGTGTGGCATCAGTGATTTACACACACCC-3': the mismatched bases are underlined) is an anti-sense oligomer with point mutations to convert Pro-355 (CCA) to Ala (GCA). Primer B (5'-GGGTGTGTGTAAATCACTGATGCCACACAATTGTAG-3': the mismatched bases are underlined) is the corresponding sense oligomer of the 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. 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.* 4). The gel revealed the mutant to be 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 kinetic experiments were carried out in triplicate. The

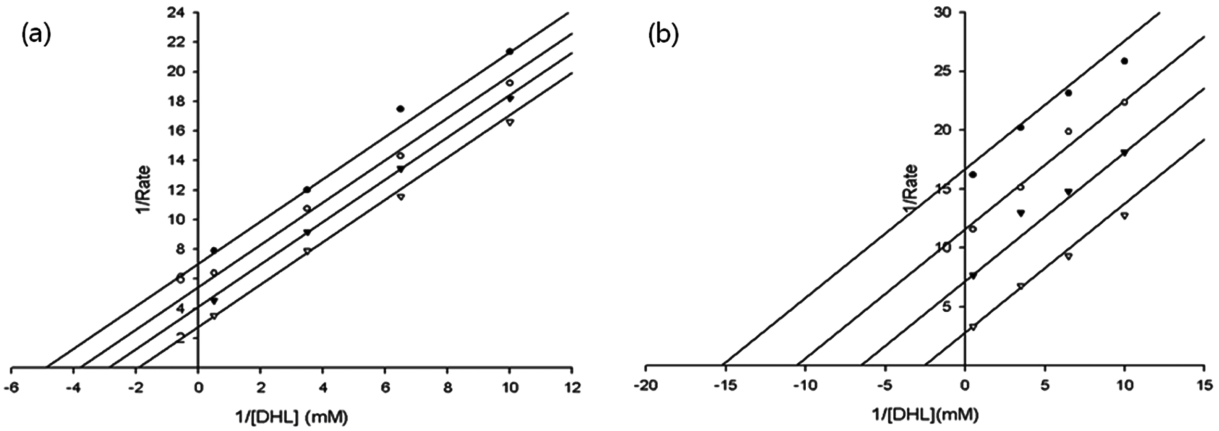

***Figure* 5.** Double reciprocal plots for the wild-type (a) and P355A (b) mutant human E3s. E3 activities were determined at 37°C in a 50 mM potassium phosphate buffer (pH 8.0) containing 1.5 mM EDTA with various concentrations of the substrates, dihydrolipoamide (DHL) and NAD^+. Plots were drawn with the SigmaPlot Enzyme Kinetics Module program. The NAD^+ concentrations from top to bottom are 0.1, 0.154, 0.286 and 2 mM. The DHL concentrations from right to left are 0.1, 0.154, 0.286 and 2 mM.

***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	899±114	0.64±0.06	0.19±0.02	1405	4732
P355A mutant	624±14	0.52±0.02	0.70±0.01	1200	891

[a]Dihydrolipoamide.

data was analyzed using the SigmaPlot Enzyme Kinetics Module (Systat Software Inc., San Jose, USA), which generated double reciprocal plots, as shown in *Fig*. 5. The plots showed parallel lines, indicating that the mutant also catalyze the reaction *via* a ping pong mechanism. 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 decreased by 30%, indicating that the mutation significantly deteriorates the catalytic processes of the conversion of substrates to products. The K_m value toward NAD^+ increased by 3.7 times, indicating that the mutation makes enzyme binding to NAD^+ substantially less efficient. The catalytic efficiency of the mutant toward NAD^+ was decreased by 81%, indicating that the mutant became a significantly inefficient enzyme toward NAD^+. The NAD^+ concentration in cells was determined to be 0.37 mM.[6,7] These significantly reduced apparent binding affinity and catalytic efficiency toward NAD^+ of the mutant could be more detrimental inside cells because of the low cellular NAD^+ concentration.

The amino acid volume of Pro is 112.7 $Å^3$, whereas that of Ala is 88.6 $Å^3$.[8] A Pro to Ala mutation will result in a vacancy of 24.1 $Å^3$ at the mutated residues, which will remove the conformational rigidity of Pro at the mutation site. This vacancy and conformation freedom will cause structural changes at the mutation sites. The van der Waals interactions between Pro-355 and His-329 are also affected. These structural changes will alter the kinetic parameters of the mutants. The effect of structural changes is not confined in the local region of the mutation site. It spreads to other regions of the mutant so that the significantly reduced apparent binding affinity and catalytic efficiency toward NAD^+ of the mutant can occur.

Fluorescence spectroscopy was performed to examine the structural changes occurring in the mutants. When the enzymes were excited at 296 nm, two fluorescence emissions were observed for the mutant and normal E3s, as shown in *Fig*. 6. The first emission from 305 nm to 400 nm was due mainly to Trp. The second emission from 480 nm to > 550 nm was assigned to FAD. In human E3, the Trp

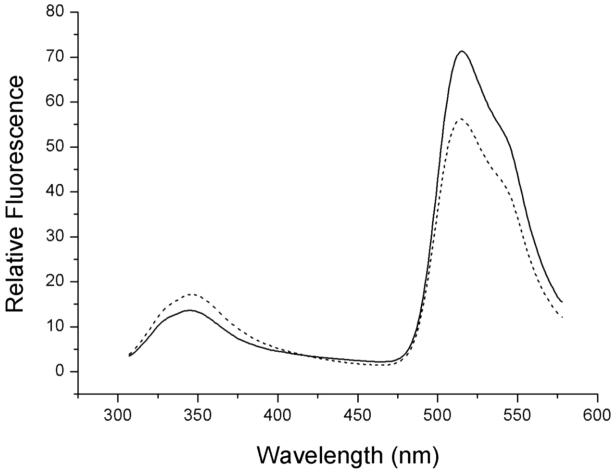

***Figure* 6.** Fluorescence spectra of the mutant (dotted line) and wild-type (solid line) human E3s. Enzymes were excited at 296 nm and the emissions were observed from 305 nm to 575 nm. The data were transferred to an ASCII file and the spectra were then drawn using the MicroCal Origin program.

fluorescence was quenched due to fluorescence resonance energy transfer (FRET) from Trp to FAD. When the fluorescence spectra were compared, there was a large difference in the ratio between the relative intensities of the first and second fluorescence emissions. The ratio (3.3) between the relative intensities of the first and second fluorescence emissions of the mutant (dotted line) was significantly smaller than that (5.2) of the normal enzyme (solid line). This suggests that the FRET from Trp to FAD was disturbed in the mutant. The Pro-355 to Ala mutation could change the structure of human E3, interfering with energy transfer from Trp to FAD. The precise structural changes occurring due to the mutation can only be revealed by an X-ray crystallographic study.

This study examined the effects of the mutations of Pro-355 to Ala in human E3 on the structure and function of the enzyme using site-directed mutagenesis, E3 activity measurements and fluorescence spectroscopy. The mutant possesses a significantly reduced k_{cat} value, indicating the mutation significantly deteriorates the catalytic power of the enzyme. The mutant shows a larger K_m values toward

NAD^+, indicating that that the mutation makes enzyme binding to NAD^+ substantially less efficient. The mutant shows significantly decreased catalytic efficiency toward NAD^+, indicating the mutation make the enzyme much less efficient toward NAD^+. The mutation of Pro-355 to Ala also results in structural changes, which interfered with the efficient FRET from Trp to FAD. These findings indicate that the conservation of Pro-355 in human E3 is very important for the proper catalytic function and structure of the enzyme.

Acknowledgements. The authors thank 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.

REFERENCES

1. Jentoft, J. E.; Shoham, M.; Hurst, D.; Patel, M. S. *Proteins: Struct. Funct. Bio.* **1992**, *14*, 88.
2. Pons, G.; Raefsky-Estrin, C.; Catothers, D. J.; Pepin, R. A.; Javed, A. A.; Jesse, B. W.; Ganapathi, M. K.; Samols, D.; Patel, M. S. *Proc. Natl. Acad. Sci. U.S.A.* **1988**, *85*, 1422.
3. Reed, L. J. *Acc. Chem. Res.* **1974**, *7*, 40.
4. Grafakou, O.; Oexle, K.; van den Heuvel, L.; Smeets, R.; Trijbels, F.; Goebel, H. H.; Bosshard, N.; Superti-Furga, A.; Steinmann, B.; Smeitink, J. *Eur. J. Pediatr.* **2003**, *162*, 714.
5. Kim, H. *J. Kor. Chem. Soc.* **2016**, *60*, 378.
6. Yamada, K.; Hara, N.; Shibata, T.; Osago, H.; Tsuchiya, M. *Anal. Biochem.* **2006**, *352*, 282.
7. Yang, H.; Yang, T.; Baur, J. A.; Perez, E.; Matsui, T.; Carmona, J. J.; Lamming, D. W.; Souza-Pinto, N. C.; Bohr, V. A.; Rosenzweig, A.; de Cabo, R.; Sauve, A. A.; Sinclair, D. A. *Cell* **2007**, *130*, 1095.
8. Zamyatin, A. A. *Prog. Biophys. Mol. Biol.* **1972**, *24*, 107.