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Notes

Characterization of Human Dihydrolipoamide Dehydrogenase Mutant with Significantly Decreased Catalytic Power

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Dihydrolipoamide dehydrogenase (E3) (dihydrolipoamide: NAD^+ oxidoreductase; EC 1.8.1.4) is an essential component in three α-keto acid dehydrogenase complexes (pyruvate, α-ketoglutarate and branched-chain α-keto acid dehydrogenase complexes)[1] and the glycine cleavage system.[2] E3 facilitates the reoxidation of the dihydrolipoyl prosthetic group of the acyltransferase components of the three α-keto acid dehydrogenase complexes and of the hydrogen-carrier protein of the glycine cleavage system. The decrease of the E3 activity can affect the activities of all three complexes, because E3 is commonly present in them. This results in increased urinary excretion of α-keto acids, elevated blood lactate, pyruvate, and branched chain amino acids. Patients with an E3 deficiency normally die young, because it is a critical genetic defect that affects parts of 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 exists as a homodimeric form containing one FAD as a prosthetic group at each subunit (*Fig.* 1).[4] Each subunit consists of 474 amino acids with a molecular mass of 50,216 daltons.[5] Along with glutathione reductase, thioredoxin reductase, mercuric reductase and trypanothione reductase, E3 belongs to the pyridine nucleotide-disulfide oxidoreductase family.[6] The catalytic mechanisms of pyridine nucleotide-disulfide oxidoreductases are similar. All of these oxidoreductases have homodimeric structures containing an active disulfide center and a FAD in their each subunit. Through the FAD and active disulfide center, the oxidoreductases catalyze electron transfers between pyridine nucleotides (NAD^+ or NADPH) and their specific substrates. The oxidoreductases consist of four structural domains (FAD, NAD, central and interface domains).[7]

Knowledge about the binding modes of FAD and NAD^+ to human E3 can be inferred from their X-ray crystallographic structure.[4] On the other hand, the binding mode of dihydrolipoamide is not well known, because the structure of human E3 with dihydrolipoamide has not been determined yet. His-329 is located at the end of the presumed dihydrolipoamide binding channel (*Fig.* 1). This suggests that His-329 may be involved in interactions with dihydrolipoamide.

Fig. 2 shows the sequence alignment of the His-329 region of human E3 with the corresponding regions of E3s from a range of sources, such as pigs, yeast, *Escherichia coli* and *Pseudomonas fluorescens*. His-329 is absolutely conserved in the various E3s including human E3, suggesting that it might be important for their structure and function. His-329 is a part of the long α-helix 8, which is composed of 16 amino acids and is a component of the central domain. His-329 is also located near FAD and the active disulfide center between Cys-45 and Cys-50, which are essential to the catalytic activity of human E3 (*Fig.* 3).

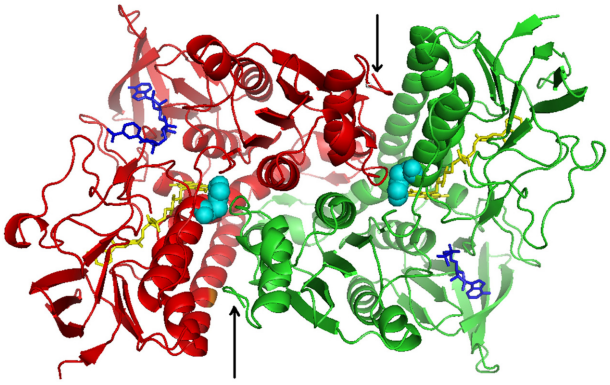

***Figure* 1.** Homodimeric structure of human E3. The two subunits are shown as cartoons, with each secondary structure portrayed in a single color (red and green, respectively). FAD (yellow) and NAD^+ (blue) are shown as sticks and His-329 (cyan) is shown as spheres. The arrows indicate the dihydrolipoamide binding channels. The structure was drawn using the PyMOL program (DeLano Scientific LLC). The PDB ID for the human E3 structure is 1ZMC.

E3	Amino acid sequence
	329
sp|P09622|DLDH_HUMAN	GDVVAGPMLAHKAEDEGII
sp|P09623|DLDH_PIG	GDVVAGPMLAHKAEDEGII
sp|P09624|DLDH_YEAST	GDVTFGPMLAHKAEEEGIA
sp|P0A9P0|DLDH_ECOLI	GDIVGQPMLAHKGVHEGHV
sp|P14218|DLDH_PSEFL	GDVVRGAMLAHKASEEGVM

	α8

***Figure* 2.** Sequence alignment of the His-329 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-319 to residue-337 are shown. H-329 and the corresponding residues are underlined. The alignment analysis was performed using the MAFFT program on the ExPASy Proteomics Server (Swiss Institute of Bioinformatics).

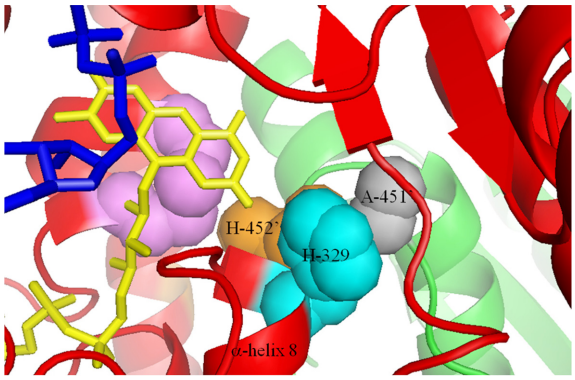

***Figure* 3.** Location of His-329 in human E3. The two subunits of human E3 are shown as cartoons, with each secondary structure portrayed in a single color (red and green, respectively). FAD (yellow) and NAD^+ (blue) are shown as sticks and the active disulfide center (violet), Ala-451' (grey), His-452' (orange) and His-329 (cyan) are shown as spheres. His-329 is located close to FAD and the active disulfide center and can form van der Waals interactions with Ala-451' and His-452' 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.

EXPERIMENTAL SECTION

Materials

E. coli XL1-Blue containing the 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 obtained from Sigma-Aldrich (St. Louis, USA). Dihydrolipoamide was synthesized by the reduction of lipoamide using sodium borohydride. Isopropyl-β-D-thiogalactopyranoside (IPTG) was obtained from Promega (Madison, USA). Ni-NTA His-Bind Resin was obtained from QIAGEN (Hilden, Germany). The primers and dNTP were obtained from Bioneer (Daejeon, Korea). The Muta-Direct™ Site-Directed Mutagenesis Kit was from iNtRON Biotechnology (Seongnam, Korea).

Site-directed mutagenesis

Site-directed mutagenesis was carried out using a mutagenesis kit. Two mutagenic primers were used for the mutations. Primer A (5'-GGTCCAATGCTGGCTGCCAAAGCAGAGGATGAAG-3': the mismatched bases are underlined) is an anti-sense oligomer with point mutations to convert His-329 (CAC) to Ala (GCC). Primer B (5'-CTTCATCCTCTGCTTTGGCAGCCAGCATTGGACC-3': the mismatched bases are underlined) is the corresponding sense oligomer of the primer A. PCRs were carried out using the human E3 expression vector pPROEX-1:E3 as a template in a programmable PCR machine. Whole DNA sequence of human E3 coding region was sequenced to verify the integrity of DNA sequences other than the anticipated mutations.

Expression and purification of the human E3 mutant

3 ml of an overnight culture of *E. coli* DH5α containing the human E3 mutant expression vector were used to inoculate 1 L of LB medium containing ampicillin (100 μg/ml). 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 shifted to 30 °C and 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 then recollected 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. The column had been washed with 2 column volumes of distilled water and then equilibrated with 5 column volumes of Binding buffer. After loading of 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.

SDS-polyacrylamide electrophoresis

SDS-PAGE analysis of 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 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 μmol 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

To examine the role and importance of His-329 in the structure and function of human E3, it was site-specifically mutated to Ala as described in *Experimental Section*. The mutant was expressed in *E. coli* by IPTG induction (1 mM). The 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 showed that the mutants were highly purified.

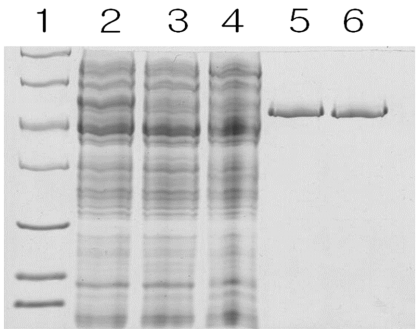

***Figure* 4.** SDS-polyacrylamide gel for the purification of the mutant E3. Lane 1, molecular weight markers (from top to bottom, b-galactosidase 116.3 kDa, bovine serum albumin 66.2 kDa, ovalalbumin 45.0 kDa, lactate dehydrogenase 35.0 kDa, REase Bsp981 25 kDa, b-lactoglobulin 18.4 kDa, lysozyme 14.4 kDa); lane 2, supernatant; lane 3, flow-through; lane 4, Binding buffer containing 50 mM imidazole; lane 5, Binding buffer containing 250 mM imidazole; lane 6, previously purified recombinant human E3 as a control.

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 the kinetic parameters. The kinetic experiments were performed in triplicate. The data was analyzed using the SigmaPlot Enzyme Kinetics Module, which generated double reciprocal plots (*Fig*. 5). The plots showed parallel lines, indicating that the mutant also catalyzes the reaction *via* a ping pong mechanism. The kinetic mechanism of human E3 is a ping-pong mechanism. The enzyme binds first to dihydrolipoamide and electrons are then transferred from dihydrolipoamide to the enzyme. This results in a two-electron reduced form of

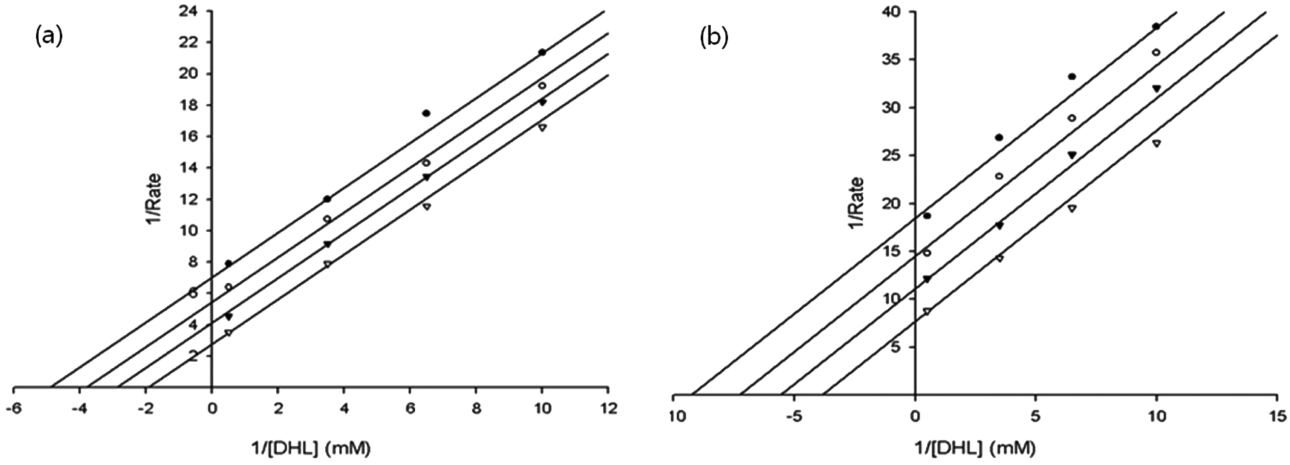

***Figure* 5.** Double reciprocal plots for the wild-type (a) and mutant (b) human E3s. The activities of E3 were determined at 37 °C in a 50 mM potassium phosphate buffer (pH 8.0) containing 1.5 mM EDTA with variable concentrations of the substrates, dihydrolipoamide (DHL) and NAD^+. The plots were drawn 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 dihydrolipoamide (mM)	K_m toward NAD^+ (mM)
Wild-type	899±114	0.64±0.06	0.19±0.02
H329A mutant	37±0	0.29±0.01	0.16±0.00

the enzyme. The enzyme expels the oxidized lipoamide and binds to NAD^+. The electrons are transferred from the enzyme to NAD^+ to produce NADH, which will finally falls off the enzyme.[9]

Table 1 lists the kinetic parameters of the mutant and wild-type human E3s. The k_{cat} value of the mutant was significantly decreased 24-fold, indicating that the mutation severely deteriorates the catalytic power of the enzyme. The K_m value toward dihydrolipoamide was also significantly reduced 2.2-fold, indicating that the mutation makes the enzyme binding to dihydrolipoamide more efficient. This indicates that His-329 is involved in the enzyme binding to dihydrolipoamide. This result is consistent with the location of His-329 being at the end of the presumed dihydrolipoamide binding channel (*Fig.* 1). On the other hand, the K_m value toward NAD^+ was similar to that of the wild-type enzyme, indicating that the mutation does not significantly affect the enzyme binding to NAD^+. His-329 is located close to FAD and the active disulfide center between Cys-45 and Cys-50 (*Fig.* 3). Therefore, a His-329 to Ala mutation could affect both FAD and the active disulfide center, which play a very important role in the catalytic function of human E3, resulting in the significant deterioration of the catalytic power of the mutant. His-329 can also form van der Waals interactions with Ala-451' and His-452' from the other subunit (*Fig.* 3). His-452 is known to play an important role in the catalytic function of human E3 by acting as a proton donor/acceptor during its catalytic process.[10] The mutation of His-329 to Ala would affect these interactions, which could be also responsible for the significantly reduced catalytic power of the mutant.

Fluorescence spectroscopy was performed to examine any structural changes occurring in the mutant. Two fluorescence emissions were observed for both the mutant and wild-type E3s after excitation at 296 nm (*Fig.* 6). The first emission in the range from 305 nm to 400 nm came from Trp. The second emission in the range from 480 nm to over 550 nm was due to FAD. The fluorescence of Trp was quenched due to the fluorescence resonance energy transfer (FRET) from Trp to FAD. When the fluorescence spectra

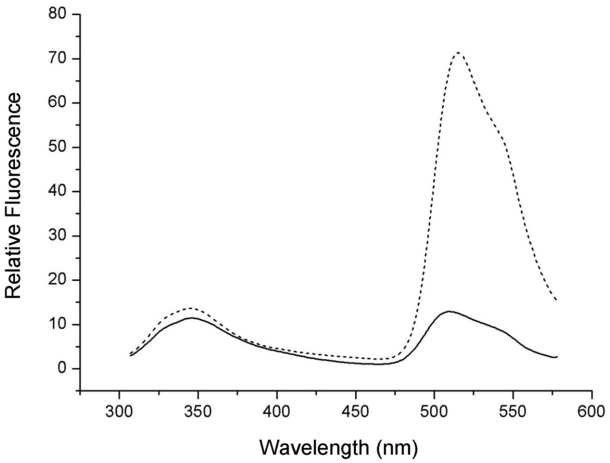

***Figure* 6.** Fluorescence spectra of the wild-type (dotted line) and mutant (solid line) recombinant human E3s. The enzymes were excited at 296 nm and the emissions were observed from 305 nm to 575 nm. The data were transferred to an ASCII file and the spectra were then drawn using the MicroCal Origin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

were compared, a noticeable difference was found in the ratio of the relative intensities of the first and second fluorescence emissions. The ratio (1.1) of the relative intensities of the first and second fluorescence emissions of the mutant (solid line) was by 4.7-fold smaller than that (5.2) of the wild-type enzyme (dotted line), indicating that the FRET was considerably disturbed in the mutant, which implies that structural changes occur within it. The amino acid volume of His is 153.2 $Å^3$, whereas that of Ala is 88.6 $Å^3$.[11] This mutation would result in a vacancy of 64.6 $Å^3$ at residue-329. The van der Waals interactions of His-329 with Ala-451' and His-452' from the other subunit (*Fig.* 3) would be also affected by this mutation. These factors could cause structural changes in human E3, which would induce alterations in the fluorescence properties and kinetic parameters of the mutant.

In this study, the effects of a site-specific mutation of His-329 to Ala in human E3 on the structure and function of the enzyme were examined by site-directed mutagenesis, the measurement of the activity of E3 and fluorescence spectroscopy. The mutation of His-329 to Ala in human E3 severely deteriorates the catalytic power of the enzyme, indicating that His-329 plays a very important role in the proper catalytic activity of human E3. This mutation also affects the enzyme binding to dihydrolipoamide, without having any significant effects on that to NAD^+, indicating that His-329 is involved in the enzyme binding to dihydrolipoamide. This result is in accordance with the location of His-329 being at the end of the presumed dihydrolipoamide binding channel. The fluorescence spectrum of the mutant

indicates that the FRET from Trp to FAD is significantly disturbed, implying that structural changes occur in the mutant. In conclusion, these findings indicate that the conservation of His-329 in human E3 is very important for the efficient catalytic activity and structure of the enzyme.

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