

## Characterization of a Site-specifically Modified Human Dihydrolipoamide Dehydrogenase Mutant Showing Significantly Changed Kinetic Properties

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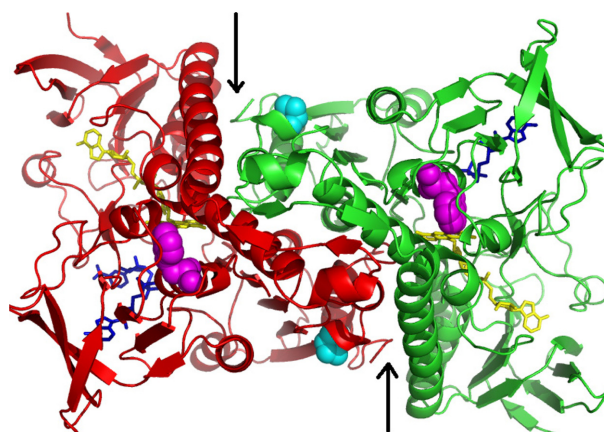
**Key words:** Dihydrolipoamide dehydrogenase, Pyridine nucleotide-disulfide oxidoreductase, Flavoenzyme

Dihydrolipoamide dehydrogenase (E3) (dihydrolipoamide:NAD<sup>+</sup> oxidoreductase; EC 1.8.1.4) is a multifunctional homodimeric flavoenzyme (Fig. 1) with each subunit composed of 474 amino acids with a molecular mass of 50,216 daltons.<sup>1</sup> E3 is an essential component in three  $\alpha$ -keto acid dehydrogenase complexes (pyruvate,  $\alpha$ -ketoglutarate and branched-chain  $\alpha$ -keto acid dehydrogenase)<sup>2</sup> and deoxidizes the dihydrolipoyl prosthetic group attached to the lysyl residue(s) of the acyltransferase components of these dehydrogenase complexes. Patients with an E3 deficiency usually die young because an E3 deficiency is a fatal genetic defect that deteriorates the central nervous system, such as the brain, which leads to serious diseases including Leigh syndrome with permanent lactic acidemia and mental retardation.<sup>3</sup>

E3 belongs to the pyridine nucleotide-disulfide oxidoreductase family along with glutathione reductase (GR), thioredoxin reductase, mercuric reductase and trypanothione reductase.<sup>4</sup> The pyridine nucleotide-disulfide oxidoreductases have similar catalytic mechanisms and structures. All of them 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 transfers between pyridine nucleotides (NAD<sup>+</sup> or NADPH) and their specific substrates. These enzymes consist of four structural domains (FAD, NAD, central and interface domains).<sup>5</sup>

Human E3 catalyses the reaction *via* a ping-pong mechanism.<sup>6</sup> Human E3 binds first to dihydrolipoamide and electrons are then transferred from dihydrolipoamide to the enzyme to make a two-electron reduced form of the enzyme. After oxidized lipoamide falls off the enzyme, NAD<sup>+</sup> then binds to the enzyme. The electrons are transferred from the enzyme to NAD<sup>+</sup> to produce NADH. NADH then falls off the enzyme and the E3 catalysis ends.

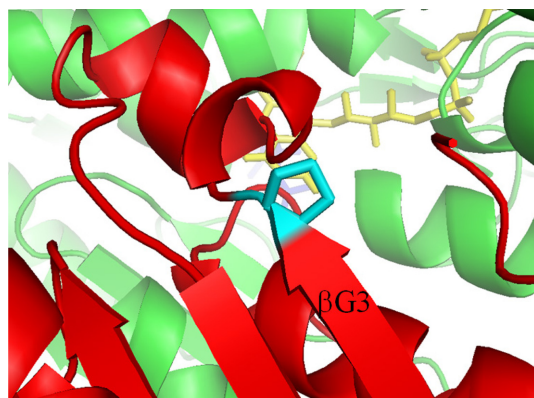
The knowledge about the binding modes of FAD and NAD<sup>+</sup> to human E3 can be speculated from the X-ray crystallographic structure.<sup>7</sup> On the other hand, the binding mode of dihydrolipoamide to the enzyme is not clearly known because the human E3 structure with dihydrolipoamide has not determined yet. Pro-387 is located close to the dihydrolipoamide binding channel (Fig. 1), suggesting that it might be involved in the dihydrolipoamide binding of the enzyme. Fig. 2 shows the sequence alignment of the Pro-387 region of human E3 with the corresponding regions of E3s from a range of sources, such as pigs, yeast, *Escherichia coli* and *Pseudomonas fluorescens*. Pro-387 is absolutely conserved in the E3s, suggesting that it might be



**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) and NAD<sup>+</sup> (blue) are shown as sticks and Pro-387(cyan) and Trp-197 (magenta) are shown as spheres. The arrows indicate the dihydrolipoamide binding channels. Dihydrolipoamide binds to the *si*-face of FAD, whereas NAD<sup>+</sup> belongs to the *re*-face. The structure was drawn using the PyMOL program (DeLano Scientific LLC). The PDB ID for the human E3 structure is 1ZMC.

E3s	Amino Acid Sequences
	387
sp P09622 DLDH_HUMAN	GIEYKVGKFPFAANSRAKT
sp P09623 DLDH_PIG	GIEYKVGKFPFAANSRAKT
sp P09624 DLDH_YEAST	GIDYKIGKFPFAANSRAKT
sp P0A9P0 DLDH_ECOLI	GISYETATFPWAASGRAIA
sp P14218 DLDH_PSEFL	GVEVNVGTFPFAASGRAMA
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	βG3            α10

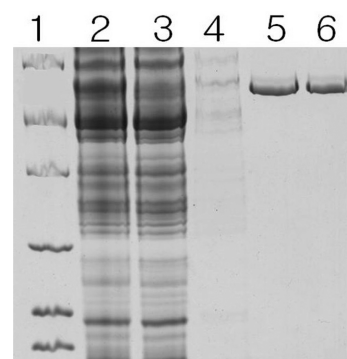
**Figure 2.** Sequence alignment of the Pro-387 region of human E3 with the corresponding regions of E3s from a range of sources (from top to bottom; human, pig, yeast, *Escherichia coli* and *Pseudomonas fluorescens*). The UniProtKB ID and amino acid sequence from residue-378 to residue-396 are shown. Pro-387 and the corresponding residues are underlined. Alignment analysis was performed using the MAFFT program on the ExPASy Proteomics Server (Swiss Institute of Bioinformatics).



**Figure 3.** Location of Pro-387 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) and Pro-387 (cyan) are shown as sticks. Pro-387 is located at the end of the β G3 secondary structure. The structure was drawn using the PyMOL program (DeLano Scientific LLC). The PDB ID for the human E3 structure is 1ZMC.

important for the structure and function of these E3s including human E3. Pro-387 acts as a β-sheet structure breaker of the short β-sheet structure G3 (Fig. 3), which is composed of 6 amino acids and a component of the interface domain.

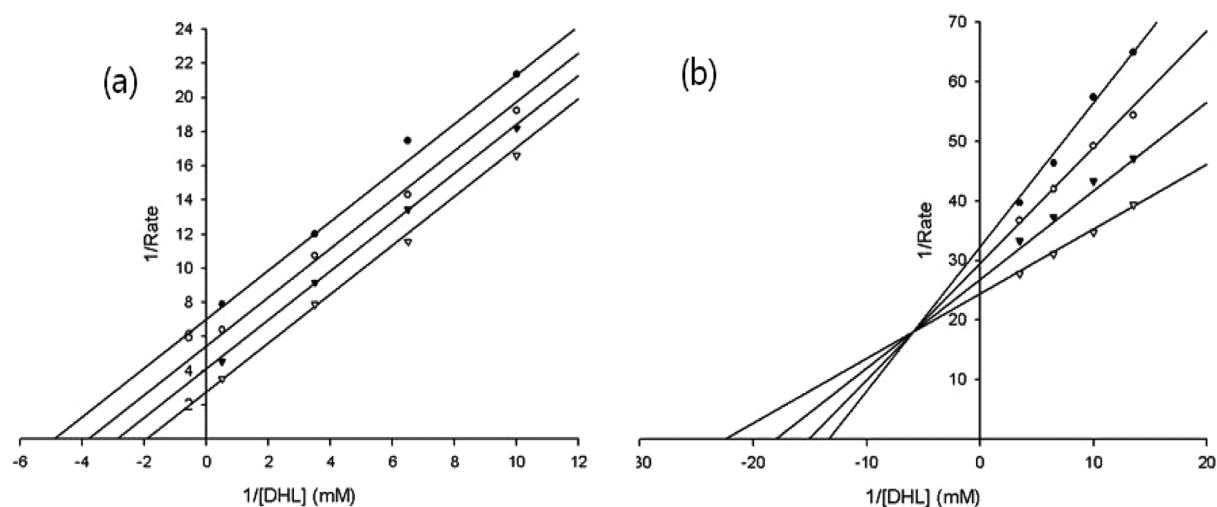
The importance of the Pro-387 residue in the human E3 structure and function was examined by a site-specific mutation to Ala. The site-directed mutation was carried out using a mutagenesis kit (iNtRON Biotechnology, Sungnam, ROK). Two mutagenic primers were used for the mutations. Primer A (5'-CAAAGTTGGGAAATTCGCATTTGCT-GCTAACAG-3': the mismatched bases are underlined) is an anti-sense oligomer with point mutations to convert Pro-387 (CCA) to Ala (GCA). Primer B (5'-CTGTTAG-



**Figure 4.** SDS-polyacrylamide gel for 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, oval-albumin 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.

CAGCAAATGCGAATTTCCCAACTTTG-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. DNA sequencing confirmed the integrity of the DNA sequences other than the anticipated mutations. The mutant was expressed with 1 mM IPTG and purified on a nickel affinity column. The purification steps were followed by SDS-polyacrylamide gel electrophoresis (Fig. 4). The gel showed that the mutant was highly purified.

An 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, dihydroli-poamide and NAD<sup>+</sup>, to determine the kinetic parameters.<sup>6</sup> The kinetic experiments were carried out in triplicate. The data was analyzed using a SigmaPlot Enzyme Kinetics. The program generated double reciprocal plots, as shown in Fig. 5. Surprisingly, the double reciprocal plot of the mutant showed a different pattern, as shown in Fig. 5(b). The plot revealed intersecting lines instead of parallel lines, as shown in Fig. 5(a), indicating that the reaction of the mutant proceeded *via* a switched kinetic mechanism rather than the ping-pong mechanism of the wild-type enzyme. The program revealed that the plot was consistent with a random sequential kinetic mechanism. The program also provided the kinetic parameters directly 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 was reduced by 7.8-fold, indicating that the mutation



**Figure 5.** Double reciprocal plots for the wild-type (a) and mutant (b) 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 variable concentrations of the substrates, dihydrolipoamide (DHL) and NAD<sup>+</sup>. Plots were drawn with the SigmaPlot Enzyme Kinetics Module program. The NAD<sup>+</sup> concentrations from top to bottom were 0.1, 0.154, 0.286 and 2 mM in (a) while those were 0.074, 0.1, 0.154 and 0.286 mM in (b). The DHL concentrations from the right to left were 0.1, 0.154, 0.286 and 2 mM in (a) while those were 0.074, 0.1, 0.154 and 0.286 mM in (b).

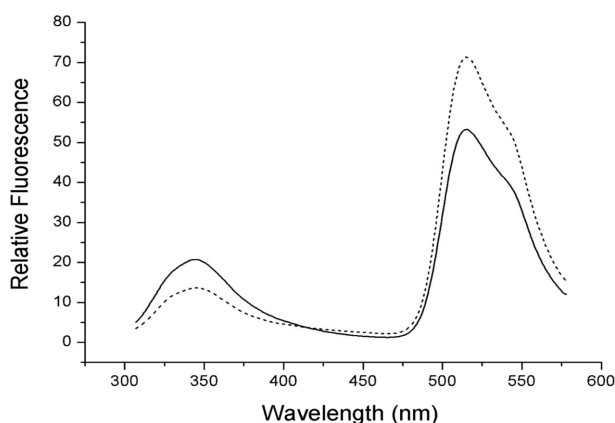
**Table 1.** Steady state kinetic parameters of mutant and wild-type human E3s. The E3 assay was performed at 37°C in a 50 mM potassium phosphate buffer (pH 8.0) containing 1.5 mM EDTA. Values are mean ± S.D. of three independent determinations

Human E3s	$k_{cat}$ (s <sup>-1</sup> )	$K_m$ toward dihydrolipoamide (mM)	$K_m$ toward NAD <sup>+</sup> (mM)
Wild-type	899 ± 114	0.64 ± 0.06	0.19 ± 0.02
P387A mutant	116 ± 3	0.031 ± 0.002	0.035 ± 0.003

deteriorated severely the catalytic power of the enzyme. On the other hand, the  $K_m$  value toward dihydrolipoamide was reduced by 20-fold, indicating that the mutation made the mutant binding to dihydrolipoamide much stronger. This can relate to the location of Pro-387, which is close to the dihydrolipoamide binding channel (Fig. 1). The  $K_m$  value toward NAD<sup>+</sup> was also reduced by 5.4-fold, indicating that the mutant binding to NAD<sup>+</sup> became significantly stronger.

In human E3, dihydrolipoamide binds to the *si*-face of FAD, whereas NAD<sup>+</sup> binds to the *re*-face, as shown in Fig. 1. These two spatially separate substrate binding sites allow the enzyme to form a ternary complex with two substrates, which is an important characteristic of the sequential mechanism. The Pro-387 to Ala mutation in human E3 can trigger this potential intrinsic property of the enzyme, resulting in a switching of the kinetic mechanism of the mutant from a ping-pong mechanism to a random sequential mechanism. The highly enhanced binding ability of the mutant toward both substrates can be responsible for the switched kinetic mechanism. Both substrates can bind simultaneously to the enzyme due to the highly enhanced the binding affinity, resulting in the formation of a ternary complex of the enzyme

with both substrates, which is a key feature of the sequential mechanism. The substantially reduced catalytic power of the mutant can be also partly responsible for the switched kinetic mechanism by delaying the changing process of substrates to products. This delay gains time for the simultaneous binding of both substrates to the enzyme, resulting in the formation of a ternary complex of the enzyme with both substrates. The switched kinetic mechanism was occurred in *E. coli* GR.<sup>8</sup> The mutations of Tyr-177 to Ser or Gly in *E. coli* GR induced a switched kinetic mechanism from a ping-pong mechanism to a sequential mechanism. The kinetic mechanisms of human and yeast GRs were observed as a mixed sequential and ping-pong mechanism. The kinetic mechanisms were dependent on the concentrations of the substrates.<sup>9</sup> It was suggested that this switchable kinetic mechanism could be applied to other pyridine nucleotide-disulfide oxidoreductases, such as E3, because they had similar structure and catalytic mechanism. The results of the present study support the suggestion. The switched kinetic mechanism was also observed in human E3 where its Ala-328 was mutated to Val.<sup>10</sup> The Val-328 mutant showed significantly decreased  $K_m$  values toward both dihydro-



**Figure 6.** Fluorescence spectra of the 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.

lipamide and  $\text{NAD}^+$  and substantially reduced catalytic power, resulting in the switched kinetic mechanism from a ping-pong mechanism to a random sequential mechanism.

The structural changes in the mutant were examined by fluorescence spectroscopy. After excitation at 296 nm, two fluorescence emissions were observed for both the mutant and wild-type E3s, as shown in Fig. 6. The first emission from 305 nm to 400 nm was attributed mainly to Trp. The second emission from 480 nm to more than 550 nm was assigned to FAD. In human E3, Trp fluorescence was quenched due to fluorescence resonance energy transfer (FRET) from Trp to FAD. A comparison of the fluorescence spectra revealed a difference in the ratio between the relative intensities of the first and second fluorescence emissions. The ratio (2.6) between the relative intensities of the first and second fluorescence emissions of the mutant (solid line) was lower than that (5.2) of the wild-type enzyme (dotted line). This suggests that FRET from Trp to FAD was disturbed in the mutant. The structural changes due to a Pro-387 to Ala mutation could have affected the structure of human E3, interfering with efficient FRET from the Trp residues to FAD.

Pro-387 is located at the end of the  $\beta$  G3 secondary structure and acts as a  $\beta$  sheet structure breaker. The Chou and Fasman secondary structure prediction method<sup>11</sup> predicts that the mutation can extend the  $\beta$  G3 secondary structure two amino acids further. This extension of the  $\beta$  sheet structure can cause structural changes. The amino acid volume of Pro was  $112.7 \text{ \AA}^3$  whereas that of Ala was  $88.6 \text{ \AA}^3$ .<sup>12</sup> A Pro to Ala mutation will result in a vacancy with a volume of  $24.1 \text{ \AA}^3$  at the residue-387, which can also remove the

conformational rigidity of Pro at the mutation site. This vacancy and conformation freedom can cause structural changes at the mutation sites, which induce alterations of the fluorescence spectrum and kinetic parameters of the enzyme, resulting in a switched kinetic mechanism. The precise structural changes due to the mutation can be revealed by an X-ray crystallographic study.

In this study, the effects of the Pro-387 to Ala mutation in human E3 on its structure and function were examined by site-directed mutagenesis, E3 activity measurements and fluorescence spectroscopy. The Pro-387 to Ala mutation triggers the potential intrinsic properties of human E3, a switchable kinetic mechanism, resulting in a change in the kinetic mechanism of the mutant from a ping-pong mechanism to a random sequential mechanism. The mutation increases significantly the binding affinity of the enzyme toward both dihydrolipoamide and  $\text{NAD}^+$ . These highly enhanced binding affinities to both substrates can lead to the formation of the ternary complex of the enzyme with the substrates, which is an essential feature of the sequential mechanism. The  $k_{cat}$  value of the mutant was reduced significantly, indicating that the mutation substantially deteriorates the catalytic power of the enzyme. This largely decreased catalytic activity can be also responsible for the switched kinetic mechanism. The mutation changes the fluorescence spectroscopic properties of the mutant, indicating that structural changes occur in the mutant. In conclusion, the conservation of the Pro-387 residue in human E3 is very important for the catalytic function and structure of the enzyme. A mutation of Pro-387 to Ala in human E3 can switch the kinetic mechanism of the enzyme from a ping-pong mechanism to a sequential mechanism.

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