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Characterization of Site-specific Human Dihydrolipoamide Dehydrogenase Mutant with a Switched Kinetic Mechanism

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Dihydrolipoamide dehydrogenase (E3) (dihydrolipoamide: NAD^+ oxidoreductase; EC 1.8.1.4) is a homodimeric flavoenzyme (Figure S1) that is a common component in three α-keto acid dehydrogenase complexes (pyruvate, α-ketoglutarate and branched-chain α-keto acid dehydrogenase complexes).[1] E3 catalyzes the reoxidation of the dihydrolipoyl prosthetic group attached to the lysyl residue(s) of the acyltransferase components of these dehydrogenase complexes. E3 belongs to the pyridine nucleotide-disulfide oxidoreductase family along with glutathione reductase (GR), thioredoxin reductase, mercuric reductase and trypanothione reductase.[2] The kinetic mechanism of human E3 is a Ping-Pong mechanism.[3]

Figure 1 shows the sequence alignment of the Ala-328 region of human E3 with the corresponding regions of the E3s from a range of sources, such as pigs, yeast, *Escherichia coli* and *Pseudomonas fluorescens*. Ala-328 is absolutely conserved, suggesting that it might be important for the structure and function of human E3. Ala-328 is a component of α-helix 8 and is located near the presumed dihydrolipoamide binding channel (Figure S1). This study examined the importance of this highly conserved Ala-328 in the human E3 catalysis and its structure by a site-directed mutation to Val. Val was selected because it causes the smallest size change among the aliphatic amino acids.

Site-directed mutagenesis was carried out using a mutagenesis kit (iNtRON Biotechnology, Sungnam, ROK). Two mutagenic primers were used for the mutation. Primer A (5'-GGTCCAATGCTGGTTCACAAAGCAGAGGATGAAG-3': the mismatched bases are underlined) is an anti-sense oligomer with point mutations to convert Ala-328 (GCT) to Val (GTT). Primer B (5'-CTTCATCCTCTGCTTTGTGAA CCAGCATTGGACC-3': the mismatched bases are underlined) is the corresponding sense oligomer of primer A. The mutant was purified using a nickel affinity column. SDS-PAGE showed that the mutant was highly purified (Figure S2).

E3s	Amino Acid Sequences 328
sp|P09622|DLDH_HUMAN	GDVVAGPML**A**HKAEDEGII
sp|P09623|DLDH_PIG	GDVVAGPML**A**HKAEDEGII
sp|P09624|DLDH_YEAST	GDVTFGPML**A**HKAEEEGIA
sp|P0A9P0|DLDH_ECOLI	GDIVGQPML**A**HKGVHEGHV
sp|P14218|DLDH_PSEFL	GDVVRGAML**A**HKASEEGVM
	----α8---

Figure 1. Sequence alignment of the Ala-328 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*). Alignment analysis was performed using the MAFFT program on the ExPASy Proteomics Server (Swiss Institute of Bioinformatics).

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^+. The data was analyzed using a SigmaPlot Enzyme Kinetics Module (Systat Software Inc., San Jose, USA). The double reciprocal plot of the mutant showed a different pattern (Figure 2(b)). The plot showed intersecting lines instead of parallel lines, indicating that the reaction proceeded *via* a switched kinetic mechanism other than the ping-pong mechanism of the normal enzyme. The plot was consistent with a random sequential kinetic mechanism with an interaction factor (α) of 8.5. The k_{cat} value of the mutant was determined to be 86 ± 4 s^{-1} which is approximately one tenth of that (899 ± 116 s^{-1}) of normal human E3, indicating that the mutation deteriorates substantially the catalytic power of human E3. The K_m value for dihydrolipoamide was determined to be 0.027 ± 0.003 mM, which was approximately 1/24 times that (0.64 ± 0.06 mM) of normal human E3, suggesting that the mutation makes enzyme binding to dihydrolipoamide much stronger. The K_m value for NAD^+ was determined to be 0.024 ± 0.004 mM, which was approximately 1/8 times that (0.19 ± 0.02 mM) of normal human E3, suggesting that the mutation makes enzyme binding to NAD^+ significantly stronger.

In human E3, dihydrolipoamide binds to the *si*-face of FAD, whereas NAD^+ binds to the *re*-face (Figure S1). These two spatially separate substrate binding sites can allow the enzyme to form a ternary complex with two substrates, which is an essential feature of the sequential mechanism. A mutation of Ala-328 to Val in human E3 triggers this potential intrinsic property of the enzyme causing the kinetic mechanism of the mutant to switch from a ping-pong mechanism to a random sequential mechanism. The highly enhanced binding ability of the mutant toward both substrates may be responsible for the switched kinetic mechanism. The switched kinetic mechanism was observed in *E. coli* GR.[4] The one amino acid substitution of Tyr-177 to Ser or Gly in *E. coli* GR resulted in a switched kinetic mechanism from a ping-pong mechanism to a sequential mechanism. The kinetic mechanisms of human and yeast GRs were determined to be a mixed sequential and

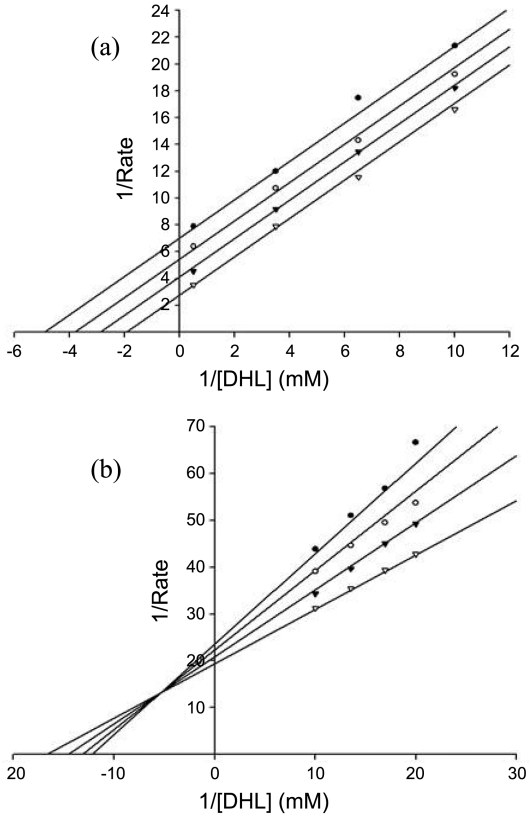

Figure 2. Double reciprocal plots for the normal (a) and mutant (b) human E3s. The E3 activities were determined at 37 °C in a 50 mM potassium phosphate buffer (pH 8.0) containing 1.5 mM EDTA with variable concentrations of the substrates, dihydrolipoamide (DHL) and NAD^+.

ping-pong mechanism depending on the concentrations of the substrates.[5] Ala-328 is also located close to the active disulfide center between Cys-45 and Cys-50 (Figure S3). An Ala-328 to Val mutation may affect the active disulfide center, which is very important to the catalytic function of human E3, resulting in a 10-fold decrease in the catalytic power of the enzyme.

Fluorescence spectroscopy was performed to examine any structural changes occurring in the mutant. When the enzymes were excited at 296 nm, two fluorescence emissions were observed for both the mutant and normal E3s (Figure 3).[6] In human E3, Trp fluorescence was quenched due to energy transfer from Trp to FAD. When the fluorescence spectra of E3s were compared, a noticeable difference was observed in the ratio between the relative intensities of the first and second fluorescence emissions. The ratio (3.2) between the relative intensities of the first and second fluorescence emissions of the mutant (solid line) was lower than that (5.2) of the normal enzyme (dotted line). This suggests that energy transfer from Trp to FAD had been disturbed in the mutant, indicating structural changes. The amino acid volume of Val is 140.0 $Å^3$, whereas that of Ala is 88.6 $Å^3$.[7] Therefore, this mutation will give an additional volume of 51.4 $Å^3$ at residue-328. A mutation of Ala-328 to Val can cause structural changes in this region, which could affect the structure of human E3

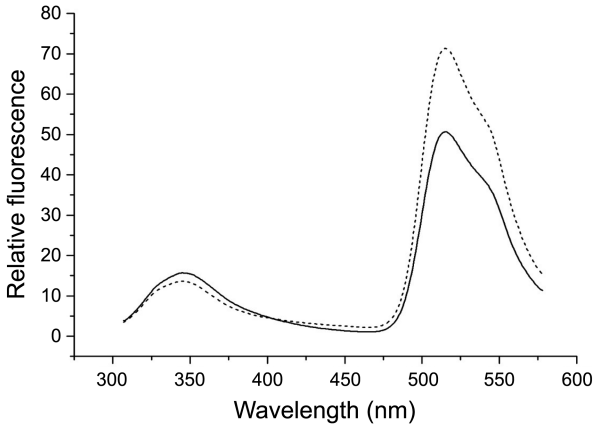

Figure 3. Fluorescence spectra of the mutant (solid line) and normal (dotted line) human E3s. The enzymes were excited at 296 nm and the emissions were observed from 305 nm to 575 nm. The data was transferred to an ASCII file and the spectra were then drawn using the MicroCal Origin program.

and interfere with energy transfer from the Trp residues to FAD. These structural changes might be responsible for the changes in the kinetic properties of the mutant.

The Ala-328 to Val mutation triggers the intrinsic potential property of human E3, the switchable kinetic mechanism, so that the kinetic mechanism of the mutant is switched from a ping-pong mechanism to a random sequential mechanism. The mutation enhances the binding affinity of the enzyme toward both substrates significantly, which may be responsible for the switched kinetic mechanism. The k_{cat} value of the mutant was reduced significantly, suggesting that the mutation also deteriorates the catalytic power of the enzyme. The mutation also changes the fluorescence spectroscopic properties of the mutant, suggesting that structural changes might occur in the mutant. In conclusion, the conservation of Ala-328 in human E3 is important for the catalytic function and structure of the enzyme. Ala-328 is essential to the normal kinetic properties of the enzyme. A mutation at Ala-328 could switch the kinetic mechanism of the enzyme from a ping-pong mechanism to a sequential mechanism.

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Supporting Information. Figures S1-3 are available in the online version of this article.

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