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Characterization of Two Site-specific Mutations in Human Dihydrolipoamide Dehydrogenase

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

Figure 1 shows the sequence alignments of the Pro-325 and Trp-366 regions of human E3 with the corresponding regions of E3s from a range of sources, such as pigs, yeast, *Escherichia coli* and *Pseudomonas fluorescens*. Pro-325 and Trp-366 are highly conserved, suggesting that they are important to the structure and function of these E3s. Pro normally plays important roles in the protein structure because it is an imino acid having conformational rigidity. Pro-325 is located at the central domain of human E3. Trp-366 is one of two Trp residues found in human E3. They are good candidates for the site-directed mutagenesis study of human E3. The aim of this research is to investigate the role and importance of Pro-325 and Trp-366 in human E3 structure and function by site-specific mutations to Ala.

The site-directed mutagenesis method is a useful tool for a structure-function study of human E3 and other proteins.[4-7] To examine the importance of Pro-325 and Trp-366 on the structure-function of human E3, they were mutated site-specifically to Ala. Two mutagenic primer pairs were used for the mutations, as listed in Table S1. The mutants were purified using a nickel affinity column, followed by SDS-polyacrylamide gel electrophoresis, as shown in Figure S2. The gel showed that the mutants were 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 a range of substrate (dihydrolipoamide and NAD^+) concentrations. The data was analyzed using the SigmaPlot Enzyme Kinetics Module (Systat Software Inc., San Jose, USA). The program generated double reciprocal plots, as shown in Figure S3. The plots showed parallel lines, indicating that both mutants catalyze the reaction through a ping pong mechanism. Table 1 lists the kinetic parameters of the mutants. The k_{cat} value of the Ala-325 mutant was approximately 150 times smaller than that of normal human E3, suggesting that

E3s	Amino Acid Sequences
	325
sp|P09622|DLDH_HUMAN	NIYAIGDVVAGPMLAHKAEDE
sp|P09623|DLDH_PIG	NIYAIGDVVAGPMLAHKAEDE
sp|P09624|DLDH_YEAST	HIKVVGDVTFGPMLAHKAEEE
sp|P0A9P0|DLDH_ECOLI	HIFAIGDIVGQPMLAHKGVHE
sp|P14218|DLDH_PSEFL	GVFAIGDVVRGAMLAHKASEE
	---> -------
	βA5 α8
	366
sp|P09622|DLDH_HUMAN	VPSVIYTHPEVAWVGKSEEQLKEEG
sp|P09623|DLDH_PIG	VPSVIYTHPEVAWVGKSEEQLKEEG
sp|P09624|DLDH_YEAST	IPSVMYSHPEVAWVGKTEEQLKEAG
sp|P0A9P0|DLDH_ECOLI	IPSIAYTEPEVAWVGLTEKEAKEKG
sp|P14218|DLDH_PSEFL	IPSVIYTHPEIAWVGKTEQTLKAEG
	--> -----> -------
	βG1 βG2 α9

Figure 1. Sequence alignment of the Pro-325 and Trp-366 region of human E3 with the corresponding regions of the 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 are shown. Pro-325, Trp-366 and the corresponding residues are underlined. Alignment analysis was performed using the MAFFT program on the ExPASy Proteomics Server (Swiss Institute of Bioinformatics).

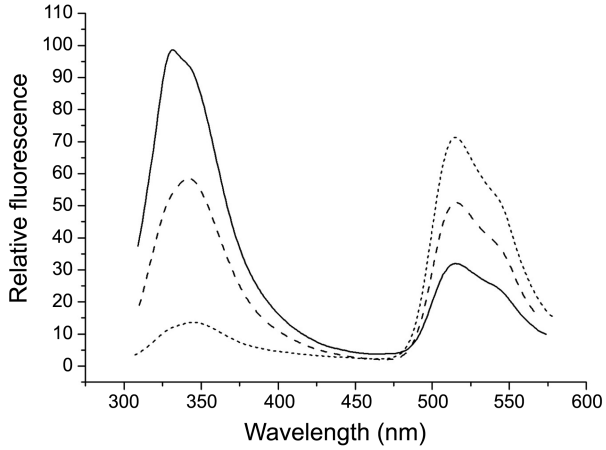

Figure 2. Fluorescence spectra of the Ala-325 mutant (solid line), Ala-366 (dashed line) and normal (dotted line) human E3s. The enzymes were excited at 296 nm and the emissions were observed from 305 nm to 575 nm. The data was transferred to an ASCII file and the spectra were then drawn using the MicroCal Origin program.

Table 1. Steady state kinetic parameters of 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 with various concentrations of the substrates. Kinetic parameters are mean ± S.D. of three independent determinations

Human E3s	k_{cat} (s^{-1})	K_m toward DHL (mM)	K_m toward NAD^+ (mM)
Normal	899 ± 114	0.64 ± 0.06	0.19 ± 0.02
Ala-325 mutant	6.0 ± 0.2	0.09 ± 0	0.06 ± 0
Ala-366 mutant	1032 ± 4	0.58 ± 0.01	0.19 ± 0.01

the mutation makes the enzyme severely less active. This indicates that Pro-325 is very important to the catalytic power of human E3. The K_m value toward dihydrolipoamide was approximately 7.1 times smaller than that of normal human E3, whereas the K_m value toward NAD^+ was approximately 3.2 times smaller. This suggests that Pro-325 is involved in enzyme binding to both substrates. The kinetic parameters of the Ala-366 mutant were comparable to those of normal human E3, suggesting that the mutation did not affect significantly the catalytic function of the enzyme. On the other hand, the amounts of the mutant (0.64 mg) after purification from a 1 L culture were 17 times smaller than those of the normal enzyme (11 mg). This suggests that Trp-366 is involved in the proper expression or folding of the enzyme in *E. coli* rather than its catalytic function.

Fluorescence spectroscopy was performed to examine the structural changes in the mutant. When the enzymes were excited at 296 nm, two fluorescence emission peaks were observed for both mutants and normal E3s, as shown in Figure 2. The first emission from 305 nm to 400 nm was assigned to Trp fluorescence. The second emission from 480 nm to > 550 nm was assigned to FAD fluorescence. In human E3, Trp fluorescence was quenched due to fluorescence resonance energy transfer (FRET) from Trp to FAD. A comparison of the fluorescence spectra of the E3s revealed a noticeable difference in the ratio between the relative intensity of the first and second fluorescence emissions. The ratio (0.3) between the relative intensities of the first and second fluorescence emissions of the Ala-325 mutant (solid line) was much smaller than that (5.2) of the normal enzyme (dotted line). This suggests that FRET from Trp to FAD was disturbed severely in the mutant. The ratio (0.9) between the relative intensities of the first and second fluorescence emissions of the Ala-366 mutant (dashed line) was also much smaller. This suggests that FRET from Trp to FAD was also disturbed severely in the Ala-366 mutant. The amino acid volume of Pro and Ala is 112.7 $Å^3$ and 88.6 $Å^3$, respectively. A Pro to Ala mutation will result in a vacancy of 24.1 $Å^3$ at the mutated residue, which will remove the conformational rigidity of Pro at the mutation site. This vacancy and conformation freedom might cause structural changes at the mutation site. The substitution of Trp-366 with Ala causes the replacement of an indole moiety (2,3-benzopyrrole) with a methyl group at residue-366. This leads to a significant decrease in amino acid volume (139.2 $Å^3$) at this site because the volume of Trp and Ala is 227.8 $Å^3$ and 88.6 $Å^3$, respectively. The structural changes due to these mutations might affect the structure of human E3, interfering with the efficient FRET from the Trp residues to FAD.

In this study, the effects of the Pro-325 to Ala and Trp-366 to Ala mutations on human E3 structure and function were examined by site-directed mutagenesis, E3 activity measurement and fluorescence spectroscopy. The mutation of Pro-325 to Ala largely affects the kinetic parameters of the enzyme. This suggests that Pro-325 is very important to the proper catalytic function of human E3. The Trp-366 to Ala mutation did not affect significantly the kinetic parameters of the enzyme. On the other hand, the mutant showed much lower expression than the normal enzyme. This suggests that Trp-366 might be important for the efficient expression or folding of the enzyme in *E. coli*. The mutations also alter the fluorescence spectroscopic properties of the mutants, suggesting that structural changes can occur in the mutants. In conclusion, the conservation of Pro-325 in human E3 is very important to the catalytic function and structure of the enzyme. Moreover, the conservation of Trp-366 in human E3 is important for the efficient expression and structure of the enzyme in *E. coli*.

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

References

1. Brautigam, C. A.; Chuang J. L.; Tomchick, D. R.; Machius, M.; Chuang, D. T. *J. Mol. Biol.* **2005**, *350*, 543.
2. Reed, L. J. *Acc. Chem. Res.* **1974**, *7*, 40.
3. Williams, C. H., Jr., *Enzymes*, 3rd ed; Boyer, P., Academic Press: 1976; p 89.
4. Yuan, L.; Cho, Y.-J.; Kim, H. *Bull. Korean Chem. Soc.* **2009**, *30*, 777.
5. Park, S. H.; Kim, B. G.; Lee, S. H.; Lim, Y.; Cheong, Y.; Ahn, J.-H. *Bull. Korean Chem. Soc*. **2007**, *28*, 2248.
6. Yoon, M.-Y.; Lee, K.-J.; Kim, J.; Park, H.-C.; Park, S.-H.; Kim, S. G.; Kim, S.-K.; Choi, J.-D. *Bull. Korean Chem. Soc.* **2009**, *30*, 1360.
7. Do, H.-D.; Jo, H.-J.; Jo, D.-H.; Kong, K.-H. *Bull. Korean Chem. Soc*. **2011**, *32*, 4106.