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Characterization of Three Site-specific Mutations in Human Dihydrolipoamide Dehydrogenase (Gly-42 to Ala, Gly-43 to Ala and Val-48 to Trp)

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Dihydrolipoamide dehydrogenase (E3) (dihydrolipoamide: NAD^+ oxidoreductase; EC 1.8.1.4) is a homodimeric enzyme that contains one FAD as a prosthetic group at each subunit. Each subunit of human E3 consists of 474 amino acids with a molecular mass of 50,216 daltons, as calculated from the primary amino acid sequence.[1] E3 is a common component in three α-keto acid dehydrogenase complexes (pyruvate, α-ketoglutarate and branched-chain α-keto acid dehydrogenase complexes)[2] and the glycine cleavage system,[3] and catalyzes the reoxidation of the dihydrolipoyl prosthetic group attached to the lysyl residue(s) of the acyltransferase components of the three α-keto acid dehydrogenase complexes and to the hydrogen-carrier protein of the glycine cleavage system. Because E3 is a common component in the three α-keto acid dehydrogenases, a decrease in E3 activity can affect the activities of all three complexes, which can result in increased urinary excretion of α-keto acids, elevated blood lactate, pyruvate and branched chain amino acids.

Table 1. Comparison of amino acid sequences around active disulfide center regions of dihydrolipoamide dehydrogenases and other pyridine nucleotide-disulfide oxidoreductases. The Gly-42, Gly-43 and Val-48 residues in human E3 and the corresponding residues in other E3s and other similar pyridine nucleotide-disulfide oxidoreductases from various sources are underlined

Enzymes/Sources	Amino acid sequence
Dihydrolipoamide dehydrogenase	
Human	GGTCLNVGCIP
Saccharomyces cerevisiae	GGTCLNVGCIP
Pseudomonas putida (*lpdg*)	GGTCLNVGCIP
Azotobacter vinelandii	GGTCLNVGCIP
Escherichia coli	GGTCLNVGCIP
Pseudomonas putida (lpdv)	GGTCLNIGCIP
Chlamydia trachomatis	GGTCLNRGCIP
Halobacterium volcanii	GGTCLNYGCIP
Bacillus subtilis (acol)	GGTCLNEGCIP
Bacillus subtilis (bfmbc)	GGTCLHKGCIP
Acholeplasma laidlawii	GGICLNHGCIP
Zymomonas mobilis	GGICLNWGCIP
Glutathione reductase	
Human	GGTCVNVGCVP
Saccharomyces cerevisiae	GGTCVNVGCVP
Escherichia coli	GGTCVNVGCVP
Spinacia oleracea	GGTCVIRGCVP
Pisum sativum	GGTCVIRGCVP
Glycine max	GGTCVIRGCVP
Mercuric reductase	
Pseudomonas fluorescens	GGTCVNIGCVP
Shigella flexneri	GGTCVNVGCVP
Alcaligenes sp.	GGTCVNVGCVP
Trypanothione reductase	
Trypanosoma brucei	GGTCVNVGCVP
Crithidia fasciculata	GGTCVNVGCVP

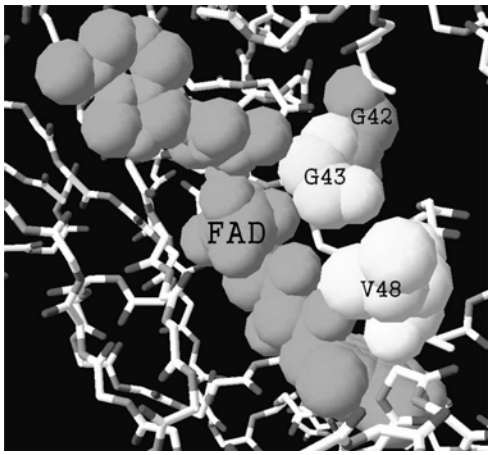

Figure 1. Location of Gly-42, Gly-43 and Val-48 in human E3. FAD, Gly-42, Gly-43 and Val-48 are shown in space-filled structures and other residues are shown in backbone structures. The structure was drawn using the Swiss-PdbViewer program (Swiss Institute of Bioinformatics). The PDB code for the human E3 structure is 1ZMC.

E3 belongs to the pyridine nucleotide-disulfide oxidoreductase family along with glutathione reductase, thioredoxin reductase, mercuric reductase and trypanothione reductase.[4] The pyridine nucleotide-disulfide oxidoreductases have similar catalytic mechanisms and structures. Their active disulfide centers play key roles in the catalyses of these enzymes. Through the FAD and active disulfide center, they catalyze electron transfer between the pyridine nucleotides (NAD^+ or NADPH) and their specific substrates. The active disulfide center regions of most pyridine nucleotide-disulfide oxidoreductases are similar, as shown in Table 1. The Gly-42 and Gly-43 residues of human E3 are absolutely conserved in all E3s and other pyridine nucleotide-disulfide

oxidoreductases from a range of sources. Val-48 is also highly conserved in many E3s. This suggests that these residues are important to the structure and function of these enzymes. These residues are also located quite close to the prosthetic group FAD, as shown in Figure 1. The importance of these residues in the human E3 structure and function was examined by site-specific mutations. Mutations of Gly-42 and Gly-43 to Ala resulted in unstable enzymes, and a mutation of Val-48 to Trp caused alterations in its kinetic parameters and spectroscopic properties. This suggests that the conservation of Gly-42, Gly-43 and Val-48 residues in human E3 are very important for its proper structure and function. This might be true for other pyridine nucleotide-disulfide oxidoreductases because they showed very good homology.

Experimental Section

Materials. The electrophoresis reagents, imidazole, iminodiacetic acid sepharose 6B, lipoamide and NAD^+ were from Sigma-Adrich (St. Louis, USA). Primers, *Pfu* polymerase, other enzymes and dNTP were obtained from Bioneer (Daejeon, Korea). Isopropyl-β-D-thiogalactopyranoside (IPTG) was obtained from KOSCHEM (Seongnam, Korea). *E. coli* XL1-Blue containing a human E3 expression vector pPROEX-1:E3 was a generous gift from Dr. Mulchand S. Patel of University at Buffalo, the State University of New York. Dihydrolipoamide was synthesized by reduction of lipoamide using sodium borohydride.

Site-Directed Mutagenesis and Construction of the Human E3 Mutant Expression Vectors. Two polymerase chain reactions (PCR) were performed using the primer pairs listed in Table 2. PCR was carried out with *Pfu* polymerase using the human E3 expression vector, pPROEX-1:E3, as a template in a programmable PCR machine. After 5 min incubation at 95 °C, thirty three rounds of temperature cycling were carried out at 95 °C for 30 sec, 43 °C for 1 min, 72 °C for 90 sec followed by a final 5 min incubation at 72 °C. As expected, PCR with primers A and B generated an approximately 220 bp fragment AB, whereas PCR with primers C and D produced and approximately 1200 bp fragment CD. Fragments AB and CD were combined and used as a template for subsequent PCR with primers A and D. After 5 min incubation at 94 °C, five rounds of temperature cycling were carried out at 94 °C for 30 sec and 72 °C for 90 sec. Thirty rounds of temperature cycling were then performed at 94 °C for 30 sec, 58 °C for 1 min, 72 °C for 90 sec and a final 5 min incubation at 72 °C. This PCR produced a 1501 bp fragment, AD, which contained the E3 sequence with the mutations.

The fragment AD was digested with *Mly*113I and *Eco*RI. The *Mly*113I/*Eco*RI fragment was then isolated by agarose gel electrophoresis. A mutant expression vector, pPROEX-1:E3(S-79→C), was digested with *Mly*113I and *Eco*RI to remove the corresponding normal *Mly*113I/*Eco*RI fragment. The vector lacking the *Mly*113I/*Eco*RI sequence was ligated with the previously isolated *Mly*113I/*Eco*RI fragment containing the mutations. The ligation resulted in the construction of mutant expression vectors, which were confirmed by DNA sequencing.

Expression and Purification of the Human E3 Mutant. 3 mL of an overnight culture of *E. coli* XL1-Blue containing the human E3 mutant expression vector was used to inoculate 600 mL of LB medium containing ampicilin (100 g/mL). The 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 the cells were cultured overnight. The overnight culture was harvested by centrifugation at 4000 × *g* for 5 min. The cell pellets were washed with 50 mM potassium phosphate buffer (pH 8.0) containing 100 mM NaCl (Binding buffer) and recollected by centrifugation at 4000 × *g* for 5 min. The resulting pellets were resuspended in 10 mL of binding buffer, lysed by sonication and centrifuged at 10,000 × *g* for 20 min.

The supernatant was loaded on a nickel iminodiacetic acid sepharose 6B column, which had been washed with 2 column volumes of distilled water and equilibrated with 5 column volumes of binding buffer. After loading the supernatant, the column was washed with 10 column volumes of binding buffer and then with the same volume of binding buffer containing 150 mM imidazole. The E3 mutant was eluted with binding buffer containing 500 mM imidazole. The purification steps were analyzed by SDS-polyacrylamide gel electrophoresis.

E3 Assay and Spectroscopic Study. The E3 activity was assayed at 37 °C in 50 mM potassium phosphate buffer (pH 8.0) containing 1.5 mM EDTA. The activity was recorded spectrophotometrically by observing the reduction of NAD^+ at 340 nm using a SPECORD200 spectrophotometer (Analytik Jena AG, Jena, USA). One unit of activity is defined as 1 μmol of NAD^+ reduced per min. The data was analyzed using the SigmaPlot Enzyme Kinetics Module (Systat Software Inc., San Jose, USA).

The UV-visible absorption spectra were recorded from 350 nm to 550 nm using the same spectrophotometer. The fluorescence spectra were recorded using a FP-6300 spectrofluorometer (Jasco Inc., Easton, USA). The samples were excited at 296 nm and the emissions were recorded from 305 nm to 580 nm. The data was transferred to an ASCII file and the spectra were drawn using the MicroCal Origin program (Photon Technology International, South Brunswick, USA).

Results and Discussion

Table 1 lists the amino acid sequences around the active site disulfide center of human E3 along with the corresponding residues of the other pyridine nucleotide-disulfide oxidoreductases from several sources. The Gly-42 and Gly-43 residues of human E3 were absolutely conserved in all known E3s and the other pyridine nucleotide-disulfide oxidoreductases from various sources, as shown in Table 1. The Val-48 in human E3 is highly conserved in these residues among the E3s and some pyridine nucleotide-disulfide oxidoreductases.

Table 2. Primers for the site-directed mutagenesis. The mismatched bases are underlined. Primer A and Primer D are sense and *anti*-sense oligomers, respectively, which can be used to amplify the whole E3 gene sequence. Primer B is an anti-sense oligomer with a point mutation. Primer C is the corresponding sense oligomer of the primer B

Mutations/ Primers	Primer Sequences
Gly-42 to Ala	
A	5'-TTACGATATCCCAACGACCG-3'
B	5'-GAGAAAAATGAAACACTTGCTGGAACATGC-TTGAATGTTG-3'
C	5'-CAACATTCAAGCATGTTCACGCAAGTGTTTC-ATTTTTCTC-3'
D	5'-GCCAAAACAAGCCAAGCTTGG-3'
Gly-43 to Ala	
A	5'-TTACGATATCCCAACGACCG-3'
B	5'-GAAAAATGAAACACTTGGTGCAACATGCTT-GAATGTTGGT-3'
C	5'-ACCAACATTCAAGCATGTTGCACCAAGTGT-TTCATTTTTC-3'
D	5'-GCCAAAACAAGCCAAGCTTGG-3'
Val-48 to Trp	
A	5'-TTACGATATCCCAACGACCG-3'
B	5'-GGTGGAACATGCTTGAATTGGGGTTGTATT-CCTTCTAAG-3'
C	5'-CTTAGAAGGAATACAACCCCAATTCAAGCA-TGTTCCACC-3'
D	5'-GCCAAAACAAGCCAAGCTTGG-3'

Site-directed mutagenesis method is a useful tool for a structure-function study of human E3 and other proteins.[5-10] Three site-specific mutations in the active disulfide center region of human E3 were performed to evaluate the importance of these residues in the structure and function of human E3. These modifications were Gly-42 to Ala, Gly-43 to Ala and Val-48 to Trp. The following two questions were addressed using these site-specific mutations in human E3. First, to what extent is the structure and function of human E3 affected by Gly-42 to Ala and Gly-43 to Ala mutations, which introduce an additional methyl group at residue-42 and 43, respectively? Second, to what extent is the structure and function of human E3 affected by the Val-47 to Trp mutation, which largely increases the volume of the amino acid at residue-47?

Site-directed mutagenesis and constructions of the mutant expression vectors were carried out using common procedures with the appropriate primers listed in Table 2. PCR was carried out using the human E3 expression vector pPROEX-1:E3 as a template in a programmable PCR machine. The whole DNA sequence of the human E3 coding region was sequenced to confirm the integrity of the DNA sequences and mutations. The expression and purification of the Ala-42 mutant was unsuccessful. The expressed mutant appeared to be quite unstable and it appeared to degrade as soon as it was expressed in *E. coli*. To examine the correctness of the expression vector component of the Gly-42 mutant expression vector, the human mitochondrial TR cDNA sequence was inserted instead of the Gly-42 mutant sequence. The human mitochondrial TR mutant was expressed and purified properly (data not shown), indicating that the expression vector component of the Gly-42 mutant expression vector did not have defects. These findings suggest that when the Gly-42 was mutated to Ala, the recombinant human E3 became too unstable to be obtained easily from *E. coli*. This indicates that Gly-42 in human E3 needs to be conserved. The Gly-42 is located quite close to the prosthetic group FAD, as shown in Figure 1. The amino acid volume of Gly was 60.1 Å^3, whereas that of Ala was 88.6 Å^3. This mutation would give an additional volume of 28.5 Å^3 at residue-42. The addition of one methyl group at residue-42 could destroy the structural stability at this FAD binding region. This might be the reason for the absolute conservation of Gly-42 in E3 and the other pyridine nucleotide-disulfide oxidoreductases.

The expression and purification of the Ala-43 mutant was also unsuccessful. The upstream sequence including the promoter and ribosome binding site was sequenced to examine the correctness of the expression vector component of the Ala-43 mutant expression vector. No mutations were observed in this sequence, indicating no defects in the expression vector component of the Ala-43 mutant expression vector. This suggests that the recombinant human E3 became too unstable to be obtained easily from *E. coli* when Gly-43 was mutated to Ala, highlighting the need to conserve Gly-43 in human E3. Gly-43 is also located very close to the prosthetic group FAD, as shown in Figure 1. The addition of one methyl group at residue-43 could also destroy the structural stability at this FAD binding region. This could be the reason for the absolute conservation of Gly-43 in E3 and the other pyridine nucleotide-disulfide oxidoreductases.

The Trp-48 mutant was expressed in *E. coli*, which is sufficient to be purified and characterized. Purification of the mutant was performed using a nickel affinity column. SDS-PAGE confirmed the purity of the mutant (data not shown). 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 data was analyzed using the SigmaPlot Enzyme Kinetics Module (Systat Software Inc., San Jose, USA). The program generated double reciprocal plots. The plot showed parallel lines, indicating that the mutant also catalyzes the reaction through a Ping Pong Bi Bi mechanism. The program also directly provides kinetic parameters without the need for secondary plots. The k_{cat} value was determined to be 733 s^{-1}, which was approximately 1.2-fold smaller than that (899 s^{-1}) of normal human E3. The K_m value toward dihydrolipoamide was determined to be 0.30 mM, which was approximately 2.1-fold smaller than that (0.64 mM) of normal human E3, indicating that the mutation makes the enzyme more efficient to dihydrolipoamide. On the other hand, the K_m value toward NAD^+ was determined to be 0.22 mM, which was similar to that (0.19 mM) of normal human E3. The

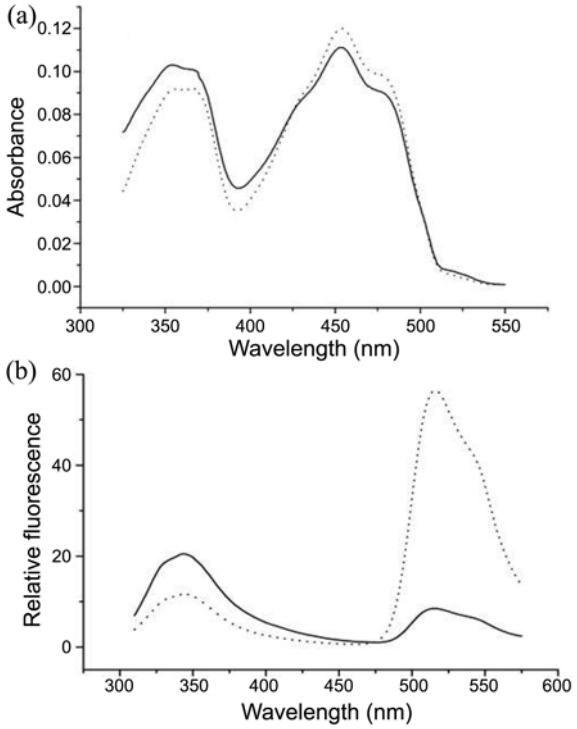

Figure 2. Spectroscopic properties of both mutant and normal human E3s. A; UV-visible spectra of the mutant (solid line) and normal (dotted line) human E3s. The spectra were recorded using a SPECORD200 spectrophotometer and the data from 325 nm to 550 nm were transferred to an ASCII file. The spectra were then drawn using the MicroCal Origin program. B; Fluorescence spectra of the mutant (solid line) and normal (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.

amino acid volume of Val was 140.0 Å^3, whereas that of Trp was 227.8 Å^3. A mutation at residue-48 mutation would provide an additional volume of 87.8 Å^3. A Val-48 to Trp mutation could cause structural changes in this region. These structural changes could be responsible for the changes in the kinetic parameters of the mutant.

UV-visible absorption and fluorescence spectroscopy were performed to examine the structural changes in the Trp-48 mutant. Human E3 has a characteristic UV-visible absorption spectrum of flavoproteins due to the prosthetic group, FAD, as shown in Figure 2(a). The mutant spectrum (solid line) had a similar overall shape to that of the normal human E3 spectrum (dotted line) but slight differences were also observed in the overall ranges of the spectra, indicating that slight structural changes might occur in the mutant. Further evidence of the slight structural changes in the mutant was provided by a fluorescence study. When the enzymes were excited at 296 nm, two fluorescence emissions were observed for both the mutant and normal E3s, as shown in Figure 2(b). The first emission from 305 nm to 400 nm was due mainly to Trp. The second emission from 480 nm to more than 550 nm was due to FAD. In human E3, the Trp fluorescence was quenched due to fluorescence resonance energy transfer (FRET) from Trp to FAD. A comparison of the fluorescence spectra revealed a large difference in the ratio between relative intensities of the first and second fluorescence emissions. The ratio (~0.4) between the relative intensities of the first and second fluorescence emissions of the mutant (solid line) was much smaller than that (~4.9) of the normal enzyme (dotted line). The increase in the intensities of the Trp fluorescence of the mutant can be attributed to the introduction of one more Trp due to the mutation of Val-48 to Trp. The intensities of the FAD fluorescence of the mutant became much smaller than those of the normal enzyme. This suggests that energy transfer from Trp to FAD was severely disturbed in the mutant. The structural changes due to the Val-48 to Trp mutation could have affected the structure of human E3, interfering with the efficient FRET from the Trp residues 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 three different mutations, Gly-42 to Ala, Gly-43 to Ala and Val-48 to Trp, in the human E3 structure and function using site-directed mutagenesis, E3 activity measurements and spectroscopic methods. The substitution of Gly-42 with Ala in human E3 was detrimental enough to destroy the stable expression of the enzyme in *E. coli*, indicating that Gly-42 must be conserved in a stable human E3 structure. The substitution of Gly-43 with Ala in human E3 also destroyed the stable expression of the enzyme in *E. coli*, indicating that the conservation of Gly-43 is also essential for a stable human E3 structure. The Val-48 to Trp mutation in human E3 resulted in slight structural changes that changed the UV-visible spectrum and interfered with the efficient FRET from the Trp residues to FAD. These structural changes can affect the kinetic parameters of the mutant. These results indicate that the conservation of Val-48 residue is important for the proper structure and function of human E3.

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