Characterization of Two Site-specifically Mutated Human Dihydrolipoamide Dehydrogenase (Leu-46 to Ala and Pro-52 to Val)

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Dihydrolipoamide dehydrogenase (E3) (dihydrolipoamide: NAD^+ oxidoreductase; EC 1.8.1.4) is a flavoenzyme.[1] It is present as a common component in three α-keto acid dehydrogenase complexes (pyruvate, α-ketoglutarate and branched-chain α-keto acid dehydrogenase complexes). It catalyzes the reoxidation of the dihydrolipoyl prosthetic group attached to the lysyl residue(s) of the acyltransferase components of these dehydrogenase complexes. It exists as a homodimeric form containing one FAD as a prosthetic group at each subunit. The subunit consists of 474 amino acids with a molecular mass of 50,216 daltons.[2]

Table 1. Comparison of amino acid sequences around active disulfide center regions of dihydrolipoamide dehydrogenases. The Leu-46 and Pro-52 residues in human E3 and the corresponding residues in other E3s are indicated by underlines.

Sources	Amino acid sequence
Human	GGTCLNVGCIP
Trypanosoma brucei	GGTCLNVGCIP
Pisum sativum	GGTCLNVGCIP
Saccharomyces cerevisiae	GGTCLNVGCIP
Pseudomonas putida (lpdg)	GGTCLNVGCIP
Azotobacter vinelandii	GGTCLNVGCIP
Escherichia coli	GGTCLNVGCIP
Sus scrofa	GGTCLNVGCIP
Canis familiaris	GGTCLNVGCIP
Mus musculus	GGTCLNVGCIP
Trypanosoma brucei	GGTCLNVGCIP
Trypanosoma cruzi	GGTCLNVGCIP
Pisum sativum	GGTCLNVGCIP
Manduca sexta	GGTCLNVGCIP
Schizosaccharomyces pombe	GGTCLNVGCIP
Pseudomonas putida (lpd3)	GGTCLNVGCMP
Pseudomonas fluoreseens	GGTCLNVGCIP
Rhodobacter capsulatus	GGTCLNVGCIP
Alcaligenes eutrophus	GGTCLNVGCIP
Haemophilus influenzae	GGVCLNVGCIP
Bacillus stearothermophilus	GGVCLNVGCIP
Bacillus subtilis	GGVCLNVGCIP
Staphylococcus aureus	GGVCLNVGCIP
Vibrio parahaemolyticus	GGVCLNVGCIP
Mycoplasma pneumoniae	GGVCLNVGCIP
Mycoplasma genitalium	GGVCLNVGCIP
Pseudomonas putida (lpdv)	GGTCLNIGCIP
Chlamydia trachomatis	GGTCLNRGCIP
Chlamydia pneumoniae	GGTCLNRGCIP
Halobacterium volcanii	GGTCLNYGCIP
Bacillus subtilis (acol)	GGTCLNEGCIP
Bacillus subtilis (bfmbc)	GGTCLHKGCIP
Acholeplasma laidlawii	GGICLNHGCIP
Zymomonas mobilis	GGICLNWGCIP
Synechocystis sp.	GGTCVNRGCIP
Chlorobium vibrioforme	GGVCVNWGCIP

Along with glutathione reductase (GR), thioredoxin reductase (TR), mercuric reductase (MR) and trypanothione reductase (TPR), it belongs to the pyridine nucleotide-disulfide oxidoreductase family.[3] These oxidoreductases have similar catalytic mechanisms and structures. Their active disulfide centers play an essential role in the catalyses of these enzymes. The active disulfide center regions of most E3s are very similar as shown in Table 1. This region of human E3 contains Leu-47 and Pro-52 residues. The Pro-52 residue is absolutely conserved in all E3s and other pyridine nucleotide-disulfide oxidoreductases from various sources. The Leu-46 residue is also highly conserved in most E3s. The conservation of these residues implies that they might be important to the structure and function of these enzymes. These residues are located close to the prosthetic group FAD as shown in Figure 1.[4] To study the importance of these residues in human E3 structure and function, they were site-specifically mutated and characterized. The mutation of the Pro-52 resulted in an unstable enzyme and that of Leu-46

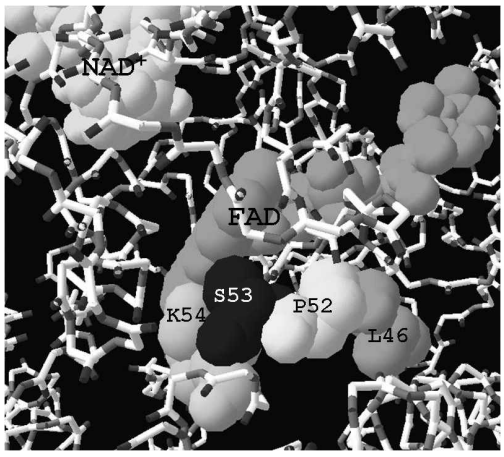

Figure 1. The location of Leu-46 and Pro-52 in a human E3 structure. FAD, NAD^+, Leu-46, Pro-52, Ser-53 and Lys-54 are shown in space-filled structures and other residues are shown in backbone structures.

showed alterations in its kinetic parameters, indicating that the conservation of Pro-42 and Leu-46 residues in human E3 was very important to its structure and function. This might be true for other pyridine nucleotide-disulfide oxidoreductases since they showed very good homology.

Experimental Section

Materials. Primers, dNTP, *Pfu* polymerase, other enzymes, primers and dNTP were obtained from Bioneer (Daejeon, Korea). The electrophoresis reagents, imidazole, iminodiacetic acid sepharose 6B, lipoamide and NAD^+ were from Sigma-Aldrich (St. Louis, USA). Isopropyl-β-D-thiogalactopyranoside (IPTG) was obtained from KOSCHEM (Seongnam, Korea). Dihydrolipoamide was synthesized by reduction of lipoamide using sodium borohydride. *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.

Site-directed mutagenesis and construction of the human E3 mutant expression vectors. Site-directed mutagenesis was performed using polymerase chain reaction (PCR) with appropriate mutagenic primers shown in Table 2. Two PCR reactions were performed using the primer pairs. They were carried out with *Pfu* polymerase using the human E3 expression vector pPROEX-1:E3 as a template in a programmable PCR machine. After a 5 min incubation at 95 °C, 33 rounds of temperature cycling were performed at 95 °C for 30 sec, 43 °C for 1 min, 72 °C for 90 sec and a final 5 min incubation at 72 °C was followed. The PCR with primers A and B generated about a 220 bp fragment AB while the PCR with primers C and D produced about a 1200 bp fragment CD. The fragments AB and CD were combined and used as a template for the subsequent PCR with primers A and D. After a 5 min incubation at 94 °C, 5 rounds of temperature cycling were performed at 94 °C for 30 sec and 72 °C for 90 sec. Thirty rounds of temperature cycling were then followed 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 was carried out. 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 and then the digested fragment was 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 the mutant expression vectors. Screening of the mutant expression vector was first performed by an *Xba*I digestion since only the mutant expression vectors had an *Xba*I site. The mutations were confirmed by DNA sequencing.

Expression and purification of the human E3 mutants. Three mL of an overnight culture of *E. coli* XL1-Blue containing the human E3 mutant expression vector were used to inoculate 600 mL of LB medium containing ampicilin (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 (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 nickel iminodiacetic acid sepharose 6B column. The column had been washed with 2 column volumes of distilled water and then equilibrated with 5 column volumes of Binding buffer. After the 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 150 mM imidazole. The mutant was eluted with Binding buffer containing 500 mM imidazole. The purification steps were analyzed by the SDS-PAGE as shown in Figure 2.

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 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).

The UV-Visible absorption spectra were recorded from 350 nm to 550 nm using the same spectrophotometer. The

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 mutations. Primer C is the corresponding sense oligomer of the primer B.

Mutations/Primers	Primer Sequences
Leu-46 to Ala	
A	5'-TTACGATATCCCAACGACCG-3'
B	5'-ACACTTGGTGGAACATGC<u>GC</u>GAATGTTGGTTGTATTCCTTC-3'
C	5'-GAAGGAATACAACCAACATT<u>CG</u>CGCATGTTCCACCAAGTGT-3'
D	5'-GCCAAAACAAGCCAAGCTTGG-3'
Pro-52 to Val	
A	5'-TTACGATATCCCAACGACCG-3'
B	5'-TGCTTGAATGTTGGTTGTATT<u>GT</u>TTCTAAGGCTTTATTGAAC-3'
C	5'-GTTCAATAAAGCCTTAGAA<u>AC</u>AATACAACCAACATTCAAGCA-3'
D	5'-GCCAAAACAAGCCAAGCTTGG-3'

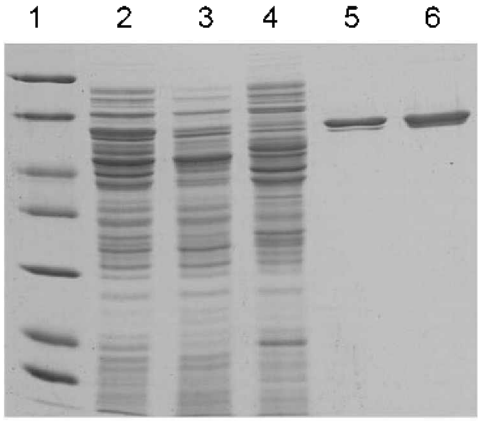

Figure 2. SDS-polyacrylamide gel for the purification of the Ala-46 mutant E3. Lane 1, molecular weight markers; lane 2, supernatant; lane 3, flow-through; lane 4, Binding buffer containing 150 mM imidazole; lane 5, binding buffer containing 500 mM imidazole; lane 6, previously purified recombinant human E3 as a control.

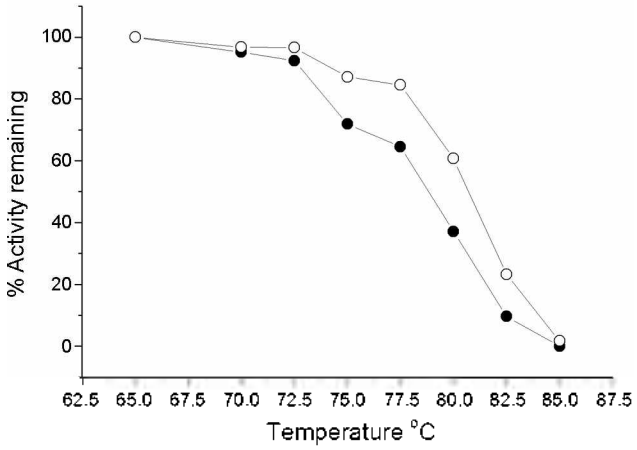

Figure 3. Thermal stability of both mutant (open circle) and wild-type (closed circle) human E3s. The enzymes were incubated at the desired temperatures for 10 min in the assay buffer and then assayed under conditions of the substrate concentrations of 2 mM dihydrolipoamide and 2 mM NAD^+.

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

Table 1 shows the comparison of amino acid sequences around the active disulfide center of human E3 and the corresponding amino acid sequences of other E3s from various sources. The Leu-46 residue in human E3, which is highlighted by an underline in Table 1, is conserved in most E3s. Only two E3s from *Synechocytis sp.* and *Chlorobium vibrioforme* have Val residue at the corresponding site. The Pro-52 residue in human E3 is absolutely conserved in all E3s and other pyridine

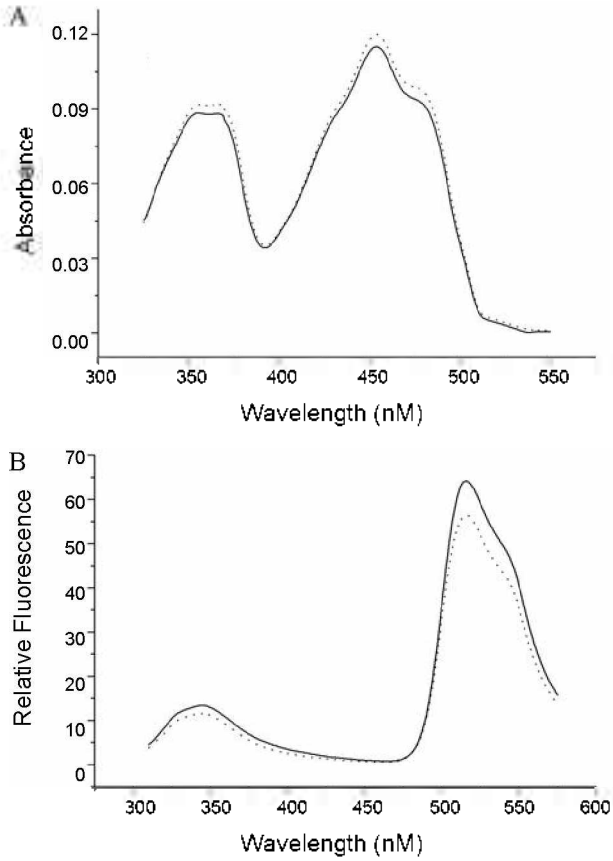

Figure 4. Spectroscopic properties of both mutant and wild-type human E3s. A; UV-Visible spectra of the mutant (solid line) and wild-type (dotted line) human E3s. B; Fluorescence spectra of the mutant (solid line) and wild-type (dotted line) recombinant human E3s.

nucleotide-disulfide oxidoreductases, implying that the Pro is very important to these enzymes. Site-directed modification method has been a helpful tool for the structure-function study of human E3 and other proteins.[5-11] To examine the importance of these residues in human E3 structure function, two site-specific mutations (Pro-52 to Val and Leu-46 to Ala) in human E3 have been made as described in the *Experimental Section*.

The purification of the Val-52 mutant was unsuccessful. The mutant was so unstable that it looked as though it became degraded as soon as it was expressed in *E. coli*. To examine the accuracy of the expression vector component of the Val-52 mutant expression vector, the upstream region of the expression vector was sequenced. The sequence was correct, indicating that the expression vector component of the Val-52 mutant expression vector did not have defects. These results implied that human E3 became too unstable to be easily obtained from *E. coli* when the Pro-52 was mutated to Val. Pro is unique since it is an imino acid. This feature gives Pro an exceptional conformational rigidity, compared to other amino acids. Pro usually plays very important roles in the protein structure such as a turn. In a human E3 structure, Pro-52 does not make direct contacts with the prosthetic group FAD.[4] However, it guides neighboring residues such as Ser-53 and Lys-54 to interact with the flavin ring of FAD as shown in Figure 1. The mutation of the Pro-52 to Val may give an additional conformational

freedom at this site, which may destroy these interactions between Ser-53, Lys-54 and FAD. The abolishment of these interactions may lead to the instability of the mutant. This may be the reason for the absolute conservation of the Pro-52, which is observed in all E3s and other pyridine nucleotide-disulfide oxidoreductases.

The Ala-46 mutant was expressed in *E. coli* by IPTG induction enough to be purified and characterized. The purification of the mutant was performed using a nickel affinity column as described in the *Experimental Section*. The SDS-PAGE showed that the mutant was 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 variable concentrations of the substrates, dihydrolipoamide and NAD^+, to determine kinetic parameters. Data was analyzed using SigmaPlot Enzyme Kinetics Module (Systat Software Inc., San Jose, USA). The k_{cat} value of Ala-46 mutant was determined as 890 s^{-1} which was similar to that of normal human E3 (899 s^{-1}). The K_m value for dihydrolipoamide was determined as 0.42 mM which was about 1.5-fold lower than that of normal human E3 (0.64 mM), indicating that the mutation makes the enzyme more efficient to dihydrolipoamide. However, the K_m value for NAD^+ was determined as 0.41 mM which was about 2.2-fold larger than that of normal human E3 (0.19 mM), indicating that the mutation makes the enzyme less efficient to NAD^+. The mutation of Leu-46 to Ala resulted in a large vacancy at the residue-46. The amino acid volume of Leu is 166.7 $Å^3$ while that of Ala is 88.6 $Å^3$. The mutation will give a vacancy of 78.1 $Å^3$ at the residue-46. This vacancy could cause a disturbance in the local structure of this region. These structural changes could be responsible for the alterations in kinetic parameters of the mutant. The thermal stability of the mutant was examined by heat treatment at the desired temperatures as shown in Figure 3. Both mutant and wild-type human E3s showed similar thermal stability profiles, indicating that the mutation did not affect the thermal stability of the enzyme. However, the mutant was slightly more stable than wild-type enzyme at high temperature. The residual activity of mutant enzyme decreased by about half at 80 °C while that of wild-type enzyme decreased by about half at 78.5 °C.

To examine any structural changes occurring in the mutant, UV-Visible absorption and fluorescence spectroscopies were performed. Due to the prosthetic group FAD, human E3 has a characteristic UV-Visible absorption spectrum of flavoproteins as shown in Figure 4A. The overall shape of the mutant spectrum (solid line) was similar to that of the wild-type human E3 spectrum (dotted line). One hint of the structural changes in the mutant came from the fluorescence study. When enzymes were excited at 296 nm, two fluorescence emissions were observed for both mutant and wild-type E3s, as shown in Figure 4B. The first emission from 305 nm to 400 nm was mainly due to Trp. The second emission from 480 nm to over 550 nm was due to FAD. In human E3, the Trp fluorescence was quenched due to energy transfer from Trp to FAD. When the fluorescence spectra were compared, a noticeable difference was found in the ratio between relative intensities of the first and second fluorescence emissions. The ratio (about 4.78) between relative intensities of the first and second fluorescence emissions of the mutant (solid line) was slightly lower than that of the wild-type enzyme (dotted line), about 4.86. This indicated that the energy transfer from Trp to FAD was slightly disturbed in the mutant. The structural changes due to the mutation of Leu to Ala could have affected the structure of human E3, interfering with the efficient energy transfer from Trp residues to FAD.

In this study, the effects of two site-specific mutations at the active site center region in human E3 structure and function were examined using site-directed mutagenesis, E3 activity measurement, heat treatment and spectroscopic methods. The substitution of Pro-52 with Val in human E3 was detrimental enough to destroy the stable expression of the enzyme in *E. coli*. This indicated that the Pro-42 residue was critical to the stability of human E3. This may be true to all other pyridine nucleotide-disulfide oxidoreductases since the residue is absolutely conserved in all these enzymes. The mutation of Leu-46 to Ala caused structural changes which slightly interfered with the efficient energy transfer from Trp residues to FAD. These structural changes could result in the altered kinetic parameters of the Ala-46 mutant. These findings indicated that the conservation of Pro-42 and Leu-46 residues was very important to the human E3 structure and function.

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