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Short communication

Activity of Human Dihydrolipoamide Dehydrogenase Is Reduced by Mutation at Threonine-44 of FAD-binding Region to Valine

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Dihydrolipoamide dehydrogenase (E3) is a member of the pyridine nucleotide-disulfide oxidoreductase family. Thr residues are highly conserved. They are at the active site disulfide-bond regions of most E3s and other oxidoreductases,. The crystal structure of *Azotobacter vinelandii* E3 suggests that the hydroxyl group of Thr that are involved in the FAD binding interact with the adenosine phosphate of FAD. However, several prokaryotic E3s have Val instead of Thr. To investigate the meaning and importance of the Thr conservation in many E3s, the corresponding residue, Thr-44, in human E3 was substituted to Val by site-directed mutagenesis. The mutant's E3 activity showed about a 2.2-fold decrease. Its UV-visible and fluorescence spectra indicated that the mutant might have a slightly different microenvironment at the FAD-binding region.

Keywords: Dihydrolipoamide dehydrogenase, Active site disulfide bond, Pyridine nucleotide-disulfide oxidoreductases, Site-directed mutagenesis, Structural homology

Introduction

Dihydrolipoamide dehydrogenase (E3) (dihydrolipoamide: NAD^+ oxidoreductase; EC 1.8.1.4) is a common component in three α-keto acid dehydrogenase complexes (pyruvate, α-ketoglutarate, and branched-chain α-keto acid dehydrogenase complexes) (Reed, 1974) and the glycine cleavage system (Walker and Oliver, 1986). It catalyzes the reoxidation of the dihydrolipoyl prosthetic group that is attached to the lysyl residue(s) of the acyltransferase components of the three α-keto acid dehydrogenase complexes, as well as to the hydrogen-carrier protein of the glycine cleavage system. It is a homodimeric flavoprotein that contains one FAD as a prosthetic group at each subunit. Each human E3 subunit consists of 474 amino acids with a molecular mass of 50,216 daltons, calculated from the primary amino acid sequence (Pons *et al.,* 1988).

E3 belongs to the pyridine nucleotide-disulfide oxidoreductase family, which also includes glutathione reductase, mercuric reductase, thioredoxin reductase, and trypanothione reductase (William, 1976). From comparison studies of the E3s structures and glutathione reductases from several sources, it becomes possible to predict the amino acid residues that are important in the human E3 function (Carothers *et al.*, 1989; Thekkumkara *et al.*, 1989). The structure of the human E3 active site has been proposed (Jentoft *et al.,* 1992) on the basis of the three-dimensional structures of the human glutathione reductase (Thieme *et al.,* 1981) and *Azotobacter vinelandii* E3 (Schierbeek *et al.,* 1989). Several site-specifically modified human E3s were made and characterized, based on these studies (Kim and Patel, 1992; Kim, 1999a; Kim, 1999b).

The catalytic mechanisms of pyridine nucleotide-disulfide oxidoreductases are similar and the disulfide bond at the active site plays a critical role in the catalysis. Cys-45 and Cys-50 have been identified as the cysteines that form a disulfide bond at the active site of human E3 (Jentoft *et al.,* 1992). The bond should be broken during the catalysis to form an intermediate with the substrate. The disulfide bond of *Azotobacter vinelandii* E3 shows an unusual structure where the distance between the C^α-C^α distance is relatively shorter than the normal value (Mattevi *et al*., 1991). The geometric strain may be necessary for the breakage of the disulfide bond during catalysis. The residues around the active site disulfide bonds of most pyridine nucleotide-disulfide oxidoreductases are highly conserved. It may be conceivable that the residues around the disulfide bond are highly conserved in order to form and keep the strain. The consensus sequence for the active site disulfide bond region of 34 E3s, which have known primary structures from various sources, was searched. The consensus sequence of GG(TV)CLN(VX)GCIP was suggested (Kim, 2001).

The crystal structure of *Azotobacter vinelandii* E3 revealed

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that the Thr-47 hydroxyl group interacts with the adenosine phosphate of FAD (Mattevi *et al*., 1991). The Thr residue is conserved in most E3s. It is also highly conserved in other homologous enzymes of the pyridine nucleotide-disulfide oxidoreductase family. However, several prokaryotic E3s have Val or Ile at the site instead of Thr. The corresponding residue, Thr-44, in human E3 was substituted to Val by site-directed mutagenesis. The E3 mutant was expressed in *Escherichia coli* and highly purified using affinity chromatography**.** The mutant showed approximately 45% E3 activity and slightly different UV-visible and fluorescence spectra, which indicates small changes in the FAD environment.

Materials and Methods

Materials. The electrophoresis reagents, imidazole, iminodiacetic acid sepharose 6B, lipoamide and NAD^+, were from the Sigma Chemical Co. (St. Louis, USA). Dihydrolipoamide was synthesized by the reduction of lipoamide using sodium borohydride. Isopropyl-β-D-thiogalactopyranoside (IPTG) was from POSCOCHEM R & D Center (Pohang, Korea). The *E. coli* XL1-Blue that contained the human E3 expression vector pPROEX-1 : E3 was a generous gift from Dr. Mulchand S. Patel of the State University of New York at Buffalo. The PCR premix, primers, and dNTP were from Bioneer (Cheongwon, Korea).

Site-directed mutagenesis and construction of the human E3 mutant expression vector pPROEX-1:E3 (T-44->V). A polymerase chain reaction (PCR) was performed with a 5' primer (5'-TTACGATATCCCAACGACCG-3'), 3' primer (5'-GCAATTC CACCTGTTAAAGC-3'), 5' mutagenic primer (5'-AATGAAACA CTTGGTGGAGTATGCTTGAATGTTGGTTGTA-3': mismatched bases are underlined), and 3' mutagenic primer (5'-TACAACCAA CATTCAAGCATACTCCACCAAGTGTTTCATT-3': mismatched bases are underlined). The reaction was carried out with a Bioneer PCR premix in a programmable PCR machine using the human E3 expression vector pPROEX-1 : E3 as a template (Cho, 2000; Kim *et al.*, 2001; Lee and Cho, 2001). After denaturation of the template DNA at 94ºC for 5 min, 35 rounds of temperature cycling were performed at 94ºC for 30 sec, 45ºC for 30 sec, 72ºC for 30 sec, followed by a final 5-min incubation at 72ºC. PCR generated a 350 bp DNA fragment that contained a human E3 sequence of which a codon (ACA) for Thr-44 was substituted to a codon (GTA) for Val. The fragment was digested with *Nar*I and *Xba*I to generate a 219 bp *Nar*I/*Xba*I fragment. The *Nar*I/*Xba*I fragment was ligated with pPROEX-1 : E3 of which *Nar*I and *Xba*I digestions removed the corresponding normal *Nar*I/*Xba*I fragment. The ligation resulted in the construction of the human E3 mutant expression vector pPROEX-1 : E3(T-44->V). The mutation was confirmed by DNA sequencing.

Expression and purification of the human E3 mutant. One ml of an overnight culture of *E. coli* XL1-Blue that contained the mutant expression vector was used to inoculate 200 ml of an LB medium that contained ampicillin (100 g/ml). The cells were grown at 37ºC to an absorbance of 0.7 at 595 nm. IPTG was added to the final concentration of 1 mM. The growing temperature was shifted to 30ºC, and the cells were allowed to grow overnight. The overnight culture was harvested by centrifugation at 4000 *g* for 5 min. The cell pellets were washed with a 50 mM potassium phosphate buffer (pH 8.0) that contained 100 mM NaCl (Binding buffer), then recollected by centrifugation at 4000 *g* for 5 min. They were resuspended in 10 ml of a binding buffer. They were lysed by a sonication treatment and centrifuged at 10,000 *g* for 20 min. The supernatant was loaded on a nickel iminodiacetic acid sepharose 6B column. The column was washed with 10 column volumes of a binding buffer, then with the same volume of a binding buffer that contained 150 mM imidazole. The E3 mutant was eluted with a binding buffer that contained 500 mM imidazole. The E3 mutant was dialyzed twice against a 10 mM potassium phosphate buffer (pH 7.5) to remove imidazole.

E3 assay and spectroscopic study. E3 activity was assayed at 37ºC in a 50 mM potassium phosphate buffer (pH 8.0) that contained 1.5 mM EDTA (Kim and Patel, 1992). The activity was recorded spectrophotometrically by observing the reduction of NAD^+ at 340 nm with a Shimazu UV 160A recording spectrophotometer. One unit of activity is defined as 1 mol of NAD^+ reduced per min/mg of protein at 37ºC.

The UV-visible absorption spectra were recorded from 250 nm to 550 nm using the same spectrophotometer. The fluorescence spectra were recorded using a Fluoromax spectrofluorometer (Industries, Inc., Edison, USA). The samples were excited at 296 nm and the emissions were recorded from 310 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 a comparison of the amino acid sequences around the active site disulfide bond regions of human E3 and the corresponding residues of other E3s from several sources. The 11 amino acids of the active site disulfide bond region of E3 are highly conserved. Among 11 amino acid residues, 6 residues are absolutely conserved. They include the 1st and 2nd Gly residues, the 4th Cys residue, the 8th Gly residue, the 9th Cys residue, and the 11th Pro residue. There are three highly-conserved residues, which are the 5th Leu, the 6th Asn, and the 10th Ile residues. There are two relatively less-conserved residues, the 3rd and 7th residues. The main amino acid for the 3rd site is Thr (68% conservation) (Table 1). The minor amino acids are Val (26%) and Ile (6%). It is interesting that the Thr residue is conserved in most eukaryotic E3s and many prokaryotic E3s, while several prokaryotic E3s (including *E. coli* E3) have Val or Ile instead of Thr. The Thr residue is also highly-conserved in the active site disulfide bond regions of the other homologous enzymes of the pyridine nucleotide-disulfide oxidoreductase family, such as glutathione reductases, mercuric reductases, and trypanothione reductases. It would be interesting to know the structural and functional effects of Thr to Val substitutions in

Table 1. Comparison of amino acid sequences around the disulfide bond at the active site of human E3 and the corresponding residues of other E3s from several sources. Bold letters highlight the Thr residue in human E3 as well as corresponding residues in other E3s.

Source	Amino acid sequence
Homo sapiens (Human)	GG**T**CLNVGCIP
Sus scrofa (Pig)	GG**T**CLNVGCIP
Canis familiaris (Dog)	GG**T**CLNVGCIP
Mus musculus (Mouse)	GG**T**CLNVGCIP
Trypanosoma brucei	GG**T**CLNVGCIP
Trypanosoma cruzi	GG**T**CLNVGCIP
Pisum sativum (Garden pea)	GG**T**CLNVGCIP
Manduca sexta (Tobacco hawk moth)	GG**T**CLNVGCIP
Saccharomyces cerevisiae (Yeast)	GG**T**CLNVGCIP
Schizosaccharomyces pombe (Fission Yeast)	GG**T**CLNVGCIP
Pseudomonas putida (*lpdg*)	GG**T**CLNVGCIP
Pseudomonas putida (lpd3)	GG**T**CLNVGCMP
Pseudomonas fluorescens	GG**T**CLNVGCIP
Azotobacter vinelandii	GG**T**CLNVGCIP
Rhodobacter capsulatus	GG**T**CLNVGCIP
Alcaligenes eutrophus	GG**T**CLNVGCIP
Pseudomonas putida (lpdv)	GG**T**CLNIGCIP
Chlamydia trachomatis	GG**T**CLNRGCIP
Chlamydia pneumoniae	GG**T**CLNRGCIP
Halobacterium volcanii	GG**T**CLNYGCIP
Bacillus subtilis (acol)	GG**T**CLNEGCIP
Bacillus subtilis (bfmbc)	GG**T**CLHKGCIP
Synechocytis sp.	GG**T**CVNRGCIP
Escherichia coli	GG**V**CLNVGCIP
Bacillus stearothermophilus	GG**V**CLNVGCIP
Bacillus subtilis	GG**V**CLNVGCIP
Staphylococcus aureus	GG**V**CLNVGCIP
Vibrio parahaemolyticus	GG**V**CLNVGCIP
Mycoplasma pneumoniae	GG**V**CLNVGCIP
Mycoplasma genitalium	GG**V**CLNVGCIP
Haemophilus influenzae	GG**V**CLNVGCIP
Chlorobium vibrioforme	GG**V**CVNWGCIP
Acholeplasma laidlawii	GG**I**CLNHGCIP
Zymomonas mobilis	GG**I**CLNWGCIP

the human E3 in order to discover the reason for the Thr conservation in human E3, as well as many other E3s.

To investigate the meaning and importance of the Thr conservation in human E3, the corresponding residue, Thr-44, in human E3 was substituted for Val by site-directed mutagenesis. The site-directed mutagenesis and construction of the human E3 mutant expression vector pPROEX-1:E3 (T-44->V) were performed, as described in *Materials and Methods*. The mutation was confirmed by DNA sequencing. The expression and purification of the mutant were performed, as described in *Materials and Methods*.

An E3 assay was performed at 37°C. The mutant E3 activity was determined as 280 unit/mg at substrate concentrations of 2 mM dihydrolipoamide and 3 mM NAD^+ at 37°C. This value was about 2.2-fold lower than that of normal human E3 activity that was determined for the same condition.

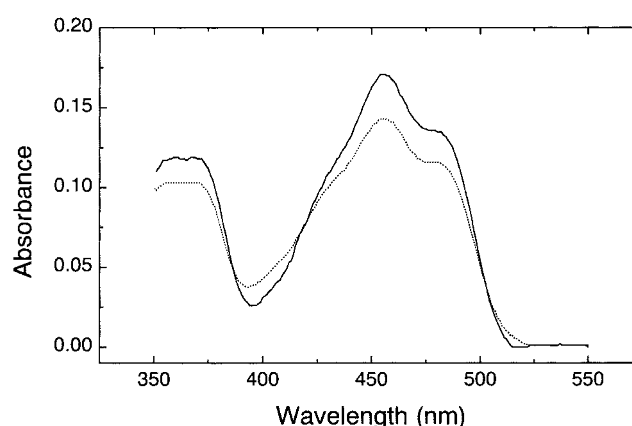

Fig. 1. UV-visible spectra of the mutant (15.1 μM, solid line) and wild-type (12.5 μM, dot line) recombinant human E3s. The spectra were recorded using a Shimazu UV 160A spectrophotometer. The data from 350 nm to 550 nm were transferred to an ASCII file. The spectra were drawn using the MicroCal Origin Program.

Due to FAD, normal E3 has characteristic UV-visible absorption spectrum patterns of flavoproteins from 350 nm to 550 nm. The spectrum of normal E3 has two peaks at 455 nm and around 365 nm (Fig. 1, dot line). To detect any changes in the UV-visible absorption spectrum pattern of the mutant E3 that occurred by the substitution, the UV-visible spectrum of the mutant E3 was recorded as described in *Materials and Methods*. As shown in Fig. 1, the UV-visible spectrum of the mutant (solid line) was slightly different from that of the normal enzyme (dot line). The mutant's valley (around 396 nm) was deeper than that of the normal enzyme. The ratio value between the mutant's peak (455 nm) and valley (396 nm) was determined as 6.8, while that of the normal enzyme was 3.7. This increased ratio value might indicate a more hydrophobic microenvironment at the FAD binding region of the mutant E3 since the ratio value that was observed in the UV-visible spectrum of FAD alone in the hydrophilic buffer solution was around 1.7. These changes in the ratio values between the peak and valley was not observed in the other mutant E3s (Kim, 1999a, b). No amino acid was modified at the active site disulfide bond region. The ratio value between the maximum absorbance value, which was around 458 nm due to FAD and that around 274 nm due to aromatic amino acid residues, is a good indicator for E3 purity. The ratio value was determined as 6.6, indicating that the purity of the mutant was very high.

Fluorescence is very sensitive to conformational changes, ligand bindings, and molecular interactions in proteins (Freifelder, 1982). When human E3s were excited at 296 nm,

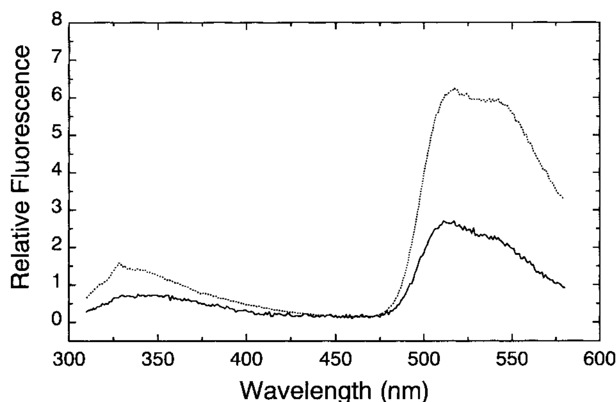

Fig. 2. Fluorescence spectra of the mutant (7.6 μM, solid line) and wild-type (12.5 μM, dot line) recombinant human E3s. The enzymes were excited at 296 nm, and the emissions were observed from 302 nm to 580 nm. The data were transferred to an ASCII file. The spectra were drawn using the MicroCal Origin Program.

two fluorescence emissions were observed in both mutant and normal enzymes (Fig. 2). The first emission was from 310 nm to 370 nm, due to aromatic amino acids; the second emission was from 480 nm to over 580 nm, due to FAD. The spectrum patterns of both mutant and normal E3s were similar considering their concentration differences. Only a slight difference was observed in the second emission, indicating small changes in the FAD environment in the mutant E3.

In the known structure of *Azotobacter vinelandii* E3, the hydroxyl group of Thr may interact with the adenosine phosphate of FAD (Mattevi *et al.,* 1991). The difference between the two amino acids, Thr and Val, is that Thr has a hydroxyl group (-OH) while Val has a methyl group (-CH_3) at their side chains. The hydroxyl group can interact with the adenosine phosphate of FAD, but the methyl group cannot. Three may be a slight size difference between the two amino acids. The substituted methyl group provides more hydrophobic microenvironment at the FAD binding region than the hydroxyl group does. These factors may result in the slight difference that is observed in the UV-visible and fluorescence spectra of the mutant E3.

As expected, the substitution of Thr-44 to Val in human E3 did not result in dramatic changes in the human E3 structure and function. However, from this study, the following reasons for the Thr conservation in the human E3, and possibly many other E3s, can be suggested. (1) The Thr-44 of the human E3 is important for the proper microenvironment at the FAD binding region by presumably making similar interactions with the adenosine phosphate of FAD, as observed in the three-dimensional structure of *Azotobacter vinelandii* E3, and (or) providing a more hydrophilic microenvironment at the FAD binding region. (2) This proper microenvironment at the FAD binding region may account for the approximate 2.2-fold efficient E3 activity of the normal human E3.

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