

Short communication

Asparagine-473 Residue Is Important to the Efficient Function of Human Dihydrolipoamide Dehydrogenase

Hakjung Kim*

Department of Chemistry, College of Natural Science, Daegu University, Kyongsan 712-714, Korea

Received 27 October 2004, Accepted 15 December 2004

Dihydrolipoamide dehydrogenase (E3) catalyzes the reoxidation of dihydrolipoyl moiety of the acyltransferase components of three α -keto acid dehydrogenase complexes and of the hydrogen-carrier protein of the glycine cleavage system. His-457 of *Pseudomonas putida* E3 is suggested to interact with the hydroxyl group of Tyr-18 of the other subunit and with Glu-446, a component in the last helical structure. To examine the importance of the suggested interactions in human E3 function, the corresponding residue of human E3, Asn-473, was substituted to Leu using site-directed mutagenesis. The E3 mutant was expressed in *Escherichia coli* and highly purified using an affinity column. Its E3 activity was decreased about 37-fold, indicating that Asn-473 residue was important to the efficient catalytic function of human E3. Its slightly altered spectroscopic properties implied that small conformational changes could occur in the E3 mutant.

Keywords: Dihydrolipoamide dehydrogenase, α -keto acid dehydrogenase complex, Pyridine nucleotide-disulfide oxidoreductase family, Site-directed mutagenesis, Structural homology

Introduction

Dihydrolipoamide dehydrogenase (E3) (dihydrolipoamide:NAD⁺ oxidoreductase; EC 1.8.1.4) is a homodimeric enzyme containing 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 calculated from the primary amino acid sequence (Pons *et al.*, 1988). 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) (Reed, 1974) and the glycine cleavage system (Walker and Oliver, 1986). It 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.

E3 along with glutathione reductase (GR), thioredoxin reductase, mercuric reductase and trypanothione reductase belong to the pyridine nucleotide-disulfide oxidoreductase family (William, 1976). All of them have homodimeric structures containing an active disulfide center and a FAD in their each subunit. Through the FAD and active disulfide center, they catalyze electron transfers between pyridine nucleotides (NAD⁺ or NADPH) and their specific substrates.

The structure of the human E3 active site has been proposed (Jentoft *et al.*, 1992) on the basis of the three-dimensional structures of human GR (Thieme *et al.*, 1981) and of *Azotobacter vinelandii* E3 (Schierbeek *et al.*, 1989). The cDNA sequence for human E3 has been cloned (Pons *et al.*, 1988) and expressed in *E. coli* (Kim *et al.*, 1991). The recombinant human E3 has possessed similar properties to those of purified mammalian E3s. The putative essential amino acid residues of human E3 have been modified using site-directed mutagenesis and the mutants have been characterized (Kim and Patel, 1992; Leu *et al.*, 1995; Kim, 2002).

The crystal structure of *Pseudomonas putida* E3 Val shows that the last five amino acid residues form an arm which is not observed in human GR (Mattevi *et al.*, 1992). The removal of the corresponding last five residues in human E3 resulted in a very low E3 activity and instability. This indicated that the last five amino acid residues were essential to the catalytic function and stable structure of human E3 (Kim, 1999b).

His-457, a component in the last helical structure, of *Pseudomonas putida* E3 is suggested to interact with the hydroxyl group of Tyr-18 of the other subunit and with Glu-446 (Mattevi *et al.*, 1992). The corresponding residue, Asn-473, of human E3 was substituted to Leu. The E3 mutant

*To whom correspondence should be addressed.
Tel: 82-53-850-6447; Fax: 82-53-850-6449
E-mail: hjkim@daegu.ac.kr

showed about 37-fold decreased E3 activity and slightly altered spectroscopic properties.

Materials and Methods

Materials. The electrophoresis reagents, imidazole, iminodiacetic acid sepharose 6B, lipoamide and NAD^+ were from Sigma Chemical Co. (St. Louis, USA). Dihydrolipoamide was synthesized by reduction of lipoamide using sodium borohydride. Isopropyl- β -D-thiogalactopyranoside (IPTG) was from POSCOCHEM R&D Center (Pohang, Korea). *E. coli* XL1-Blue containing a human E3 expression vector pPROEX-1:E3 was a generous gift from Dr. Mulchand S. Patel of State University of New York at Buffalo. Vent polymerase and T4 DNA ligase were from New England Biolab (Beverly, USA). Primers and dNTP were from Bioneer (Cheongwon, Korea).

Site-directed mutagenesis and construction of the human E3 mutant expression vector pPROEX-1:E3 (N-473->L) Polymerase chain reaction (PCR) was performed with 5' primer (5'-TCCCAAC GACCGAAAACCTG-3') and 3' mutagenic primer (5'-GCATGC **CTCGAGGGA**ACCTCAAAAGAGGATTGAT-3': mismatched bases are underlined and *XhoI* sequence is highlighted with bold letters). The reaction was carried out with Vent polymerase in a programmable PCR machine using the human E3 expression vector pPROEX-1:E3 as a template (Khumthong *et al.*, 2002; Kim *et al.*, 2002; Park and Cho, 2002). After denaturation of the template DNA at 95°C for 2 min, 33 rounds of temperature cycling were performed at 95°C for 30 s, 43°C for 1 min, 72°C for 90 s and a final 7-min incubation at 72°C was followed. The PCR generated a 1478-bp DNA fragment containing human E3 sequence of which a codon (AAC) for Asn-473 was substituted to a codon (CTC) for Leu. The fragment was digested with *EcoRI* and *XhoI* to generate a 519-bp *EcoRI/XhoI* fragment. The *EcoRI/XhoI* fragment was ligated with pPROEX-1:E3 of which the corresponding normal *EcoRI/XhoI* fragment had been removed by *EcoRI* and *XhoI* digestions. The ligation resulted in the construction of the human E3 mutant expression vector pPROEX-1:E3(N-473->L). 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 containing the mutant expression vector was used to inoculate 200 ml of LB medium containing ampicillin (100 $\mu\text{g}/\text{ml}$). 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 (Kim, 1999a). The growing temperature was shifted to 30°C and cells were allowed to grow overnight. The overnight culture was harvested by centrifugation at $4,000 \times g$ for 5 min. Cell pellets were washed with 50 mM potassium phosphate buffer (pH 8.0) containing 100 mM NaCl (Binding buffer) and then recollected by centrifugation at $4,000 \times g$ for 5 min. They were resuspended in 10 ml of Binding buffer. They were lysed by a sonication treatment and centrifuged at $10,000 \times g$ for 20 min. The supernatant was loaded on a nickel iminodiacetic acid sepharose 6B column. The column was washed with 10 column volumes of Binding buffer and then with the same volume of Binding buffer

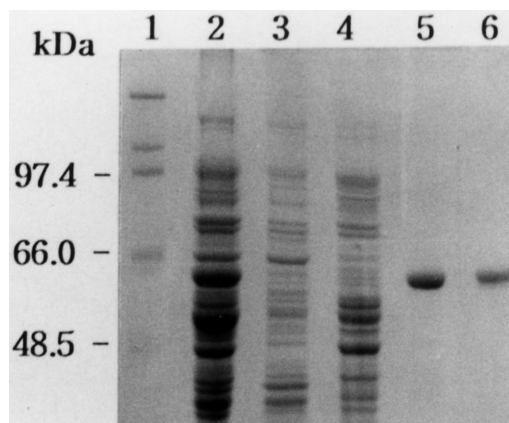


Fig. 1. SDS-polyacrylamide gel for the purification of the human E3 mutant. Lane 1, molecular weight marker (from bottom to top, bovine erythrocytes carbonic anhydrase, porcine heart fumarase, bovine serum albumin, rabbit muscle phosphorylase b; lane 2, supernatant; lane 3, flow-through; lane 4, Binding buffer containing 150 mM imidazole; lane 5, Binding buffer containing 500 mM imidazole; lane 6, previously purified normal recombinant human E3 as a control.

containing 150 mM imidazole. The E3 mutant was eluted with Binding buffer containing 500 mM imidazole. The purification steps were analyzed by the SDS-polyacrylamide gel electrophoresis as shown in Fig. 1.

E3 assay and spectroscopic study 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 Shimadzu UV 160A recording spectrophotometer. One unit of activity is defined as 1 mol of NAD^+ reduced per min/mg of protein at 37°C.

The UV-visible absorption spectrum was recorded using the Shimadzu UV 160A recording spectrophotometer from 200 nm to 650 nm. The fluorescence spectra were recorded using a Fluoromax spectrofluorometer (Industries Inc., Edison, NJ, USA). E3 was excited at 296 nm and the emission was recorded from 302 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, NJ, USA).

Results and Discussion

The carboxy-terminus regions of E3s from several sources and human GR are shown in Table 1. The crystal structure of *P. putida* E3 Val shows that the last five amino acids form an arm (Mattevi *et al.*, 1992). GR, having a good structural homology to E3s, lacks the corresponding carboxy-terminus region. The removal of the corresponding last five amino acids in human E3 led to a very low E3 activity (0.17%) and altered spectroscopic properties (Kim, 1999b). These results indicated that the last five amino acids were critical to the catalytic function and stable structure of human E3.

Table 1. Comparison of amino acid sequences at carboxy-terminus region of E3s from several sources and human glutathione reductase (GR). The Asn-473 residue in human E3 and the corresponding residues in other E3s are highlighted by italic bold letters

Protein	Source	Amino acid sequence
E3	<i>Pseudomonas putida</i>	446-EAALRALGHAL HI -458
E3	Yeast	466-ANMAAYD-KAI HC -478
E3	Pig	462-ANLAASFGKAI NF -474
E3	Human	462-ANLAASFGK SINF -474
GR	Human	477-LR-478

His-457 of *P. putida* E3 Val, a component of the last five amino acids, has been suggested to be important in the structure and function of the enzyme by forming a weak hydrogen bonding with the hydroxyl group of Tyr-18 of the other subunit (Mattevi *et al.*, 1992). It is also suggested to interact with Glu-446, a component in the last helical structure. To examine the importance of the suggested interactions in human E3 function, the corresponding residue in human E3, Asn-473, was substituted to Leu having a similar occupying volume. The substitution was expected to destroy the ability of Asn-473 forming the presumed hydrogen bonding interactions.

Site-directed mutagenesis and construction of the mutant expression vector were performed as described in *Materials and Methods*. The PCR generated a 1478 bp E3 DNA fragment in which codon (AAC) for Asn-473 was substituted to a codon (CTC) for Leu. The fragment was digested with *EcoRI* and *XhoI* and the resulting 519 bp *EcoRI/XhoI* fragment with the mutations was isolated by agarose gel electrophoresis. The human E3 expression vector pPROEX-1:E3 was digested with *EcoRI* and *XhoI* to remove the corresponding normal *EcoRI/XhoI* fragment. The resulting human E3 expression vector lacking the normal *EcoRI/XhoI* fragment was isolated by agarose gel electrophoresis. The isolated vector was ligated with the previously isolated *EcoRI/XhoI* fragment, resulting in the construction of the mutant expression vector pPROEX-1:E3(N-473->L). This process also removed the pre-existing *BamHI* site. The removal of the *BamHI* site was used for the screening of the mutant expression vector. The mutations were also confirmed by DNA sequencing.

The expression and purification of the mutant were performed as described in *Materials and Methods*. After equilibration with Binding buffer, the supernatant solution was applied to the column. *E. coli* proteins were washed out with Binding buffer and then with Binding buffer containing 150 mM imidazole. Since E3 contains FAD as a prosthetic group, it has a bright yellow color. The bright yellow color became clearer as *E. coli* proteins were washed out. This color was a good indicator for the migration of the mutant through the column. The mutant was eluted with Binding buffer containing 500 mM imidazole. Purification was followed by

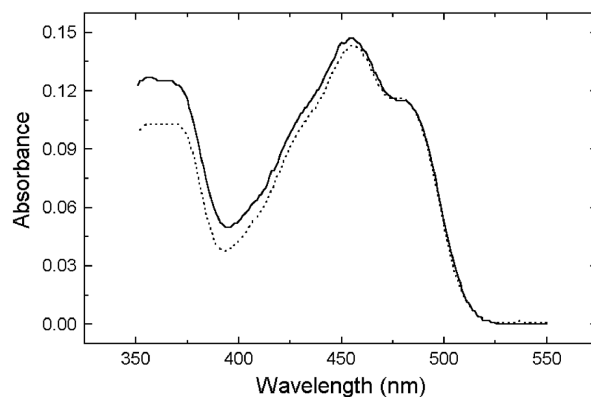


Fig. 2. UV-visible spectra of the Leu-473 mutant (13.0 μ M, solid line) and normal (12.5 μ M, dot line) recombinant human E3s. The spectra were recorded using Shimadzu UV 160A spectrophotometer and the data from 350 nm to 550 nm were transferred to an ASCII file and the spectra were drawn using the MicroCal Origin program.

SDS-polyacrylamide gel electrophoresis as shown in Fig. 1. The gel showed that the E3 mutant was highly purified.

The E3 activity of the mutant was determined as 16.6 unit/mg at substrate concentrations of 2 mM dihydrolipoamide and 3 mM NAD⁺ at 37°C. This value was about 37-fold lower than that of normal human E3 activity determined at the same condition. Even though it was difficult to obtain the detailed kinetic parameters due to the low activity and instability of the mutant, this 37-fold decreased E3 activity of the Leu-473 mutant clearly indicated that the Asn-473 residue of human E3 was important to the efficient catalytic function of the enzyme.

To examine any conformational changes in the mutant due to the substitution, UV-visible absorption and fluorescence spectra were observed as described in *Materials and Methods*. Human E3 contains one FAD as a prosthetic group in each subunit. It, therefore, shows a characteristic UV-visible absorption spectrum of flavoproteins as shown in Fig. 2 (dot line). It has two peaks, one broad peak from 358 nm to 370 nm and the other at 455 nm. There is a unique shoulder between 465 nm and 485 nm which has been observed in many flavoproteins. The strong association of FAD with the flavoproteins results in these characteristic features in the spectrum. The overall shape of the mutant spectrum (solid line) was similar to that of the normal recombinant human E3 spectrum (dot line). However, an alteration was found in the region of the peak (358 nm to 370 nm). The absorption intensity of the E3 mutant in this region was slightly higher than that of the normal enzyme. This difference in the absorption spectra implied that small conformational changes could occur in the mutant. The changes could affect the association of FAD with the enzyme so that the alteration at the peak (358 nm to 370 nm) in the UV-visible spectrum of the mutant could occur.

Another evidence for the small conformational changes in

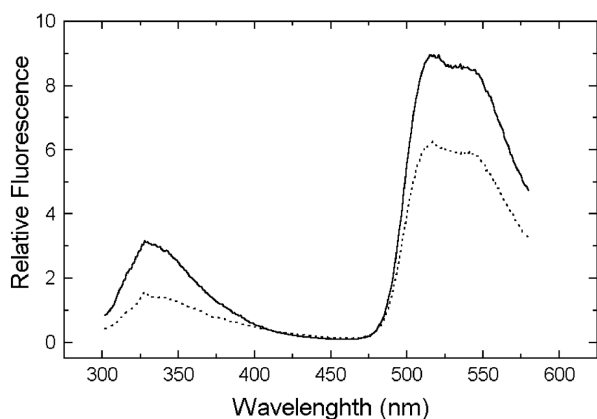


Fig. 3. Fluorescence spectra of the Leu-473 mutant (13.0 μ M, solid line) and normal (12.5 μ M, dot line) recombinant human E3s. Enzymes were excited at 296 nm and the emissions were observed from 302 nm to 580 nm. The data were transferred to an ASCII file and the spectra were drawn using the MicroCal Origin program.

the mutant came from the fluorescence study, which was very sensitive to any structural changes in proteins. E3s were excited at 296 nm and the fluorescence emissions were observed from 302 nm to 580 nm. As shown in Fig. 3, two fluorescence emissions were observed for both E3s. The first emission from 300 nm to 400 nm was due to aromatic amino acids, mainly tryptophans. The second emission from 480 nm to over 550 nm was due to FAD. When the fluorescence spectra of E3s were compared, a noticeable difference was found in the ratio between relative intensities of the first and second fluorescence emissions. The ratio (about 3) between relative intensities of the first and second fluorescence emissions of the mutant (solid line) was slightly lower than that (about 4) of the normal enzyme (dot line). This difference in the fluorescence spectra implied again that the small conformational changes could occur in the mutant. The substitution of Asn-473 to Leu could also provide more hydrophobic environments in the active site region of the mutant. This more hydrophobic environment might also partly contribute to the slightly changed fluorescence spectrum pattern of the mutant.

From these results, the following suggestions can be made. First, the Asn-473 residue is important to the efficient function of human E3, by presumably making similar hydrogen bonding interactions with the neighboring amino acids as observed in the three-dimensional structure of *P. putida* E3 Val. Second, the removal of these interactions by the substitution of Asn-473 to Leu could cause the small conformational changes in human E3.

Acknowledgments The author thanks Dr. Mulchand S. Patel (State University of New York, Buffalo) for a generous gift of an *E. coli* XL1-Blue containing a human E3 expression vector. The author is grateful to Dr. Tai Jong Kang (Daegu

University) for providing a fluorometer. This research was supported in part by the Daegu University Research Grant, 2003.

References

- Jentoft, J. E., Shoham, M., Hurst, D. and Patel, M. S. (1992) A structural model for human dihydrolipoamide dehydrogenase. *Proteins* **14**, 88-101.
- Khumthong, R., Angsuthanasombat, C., Panyim, S. and Katzenmeier, G. (2002) In vitro determination of Dengue virus type 2 NS2B-NS3 protease activity with fluorescent peptide substrates. *J. Biochem. Mol. Biol.* **35**, 206-212.
- Kim, H (1999a) Protein engineering of an artificial intersubunit disulfide bond linkage in human dihydrolipoamide dehydrogenase. *J. Biochem. Mol. Biol.* **32**, 76-81.
- Kim, H (1999b) Deletion of the last five amino acid residues in human dihydrolipoamide dehydrogenase. *Bull. Korean Chem. Soc.* **20**, 1221-1224.
- Kim, H (2002) Activity of human dihydrolipoamide dehydrogenase is reduced by mutation at threonine-44 of FAD-binding region to Valine. *J. Biochem. Mol. Biol.* **35**, 437-441.
- Kim, Y-S., Ha, K-S., Kim, Y-H. and Bae, Y-S. (2002) The ring-H2 finger motif of CKBBP1/SAG is necessary for interaction with protein kinase CKII and optimal cell proliferation. *J. Biochem. Mol. Biol.* **35**, 629-636.
- Kim, H., Liu, T-C. and Patel, M. S. (1991) Expression of cDNA sequences encoding mature and precursor forms of human dihydrolipoamide dehydrogenase in *Escherichia coli*. *J. Biol. Chem.* **266**, 9367-9373.
- Kim, H. and Patel, M. S. (1992) Characterization of two site-specifically mutated human dihydrolipoamide dehydrogenases (His-452 \rightarrow Gln and Glu-457 \rightarrow Gln). *J. Biol. Chem.* **267**, 5128-5132.
- Liu, T., Korotchkina, L. G., Hyatt, S. L., Vettakkorumakankav, N. N. and Patel, M. S. (1995) Spectroscopic studies of the characterization of recombinant human dihydrolipoamide dehydrogenase and its site-directed mutants. *J. Biol. Chem.* **270**, 15545-15550.
- Mattevi, A., Obmolova, G., Sokatch, J. R., Betzel, C. and Hol, W. G. J. (1992) The refined crystal structure of Pseudomonas putide lipoamide dehydrogenase complexed with NAD⁺ at 2.45 Å resolution. *Proteins* **13**, 336-351
- Park, S-J and Cho, Y-D. (2002) Structural roles of cysteine 50 and cysteine 230 residues in *Arabidopsis thaliana* S-adenosylmethionine decarboxylase. *J. Biochem. Mol. Biol.* **35**, 178-185.
- Pons, G., Raefsky-Estrin, C., Catothers, D. J., Pepin, R. A., Javed, A. A., Jesse, B. W., Ganapathi, M. K., Samols, D. and Patel, M. S. (1988) Cloning and cDNA sequence of the dihydrolipoamide dehydrogenase component of human aketoacid dehydrogenase complexes. *Proc. Natl. Acad. Sci. USA* **85**, 1422-1426.
- Reed, L. J. (1974) Multienzyme complexes. *Acc. Chem. Res.* **7**, 40-46.
- Schierbeek, A. J., Swarte, M. B. A., Dijkstra, B. W., Vriend, G., Reed, R. J., Hol, W. G. J. and Drenth, J. (1989) X-ray structure of lipoamide dehydrogenase from *Azotobacter vinelandii* determined by a combination of molecular and

- isomorphous replacement techniques. *J. Mol. Biol.* **206**, 365-379.
- Thieme, R., Pai, E. F., Schirmer, R. H. and Schulz, G. E. (1981) Three-dimensional structure of glutathione reductase at 2 Å resolution. *J. Mol. Biol.* **152**, 763-782.
- Walker, J. L. and Oliver, D. J. (1986) Glycine decarboxylase multienzyme complex. Purification and partial characterization from leaf mitochondria. *J. Biol. Chem.* **261**, 2214-2221.
- Williams, C. H., Jr. (1976) Flavin containing dehydrogenases; in *Enzymes*, Boyer, P. (ed.), pp. 89-173, Academic Press, New York, USA.