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

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Dihydrolipoamide dehydrogenase (E3) catalyzes the reoxidation of dihydrolipoyl moiety of the acyltransferase components of three \( \alpha \)-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, \( \alpha \)-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 \( \alpha \)-keto acid dehydrogenase complexes (pyruvate, \( \alpha \)-ketoglutarate and branched-chain \( \alpha \)-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 \( \alpha \)-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...
showed about 37-fold decreased E3 activity and slightly altered spectroscopic properties.

Materials and Methods

Materials. The electrophoresis reagents, imidazole, iminodiacetic acid sepharose 6B, lipoiamide and NAD⁺ were from Sigma Chemical Co. (St. Louis, USA). Dihydrolipoamide was synthesized by reduction of lipoiamide 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 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'-GCATGGCTCGAGGGGAACCTG-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 (Khunthong 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 and construction in 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 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 (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 × 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 × 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 × 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 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 Shimazu 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 Shimazu UV 160A recording spectrophotometer from 200 nm to 650 nm. The fluorescence spectra were recorded using a Fluoromax spectrophorometer (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.
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 *Eco*RI and *Xho*I and the resulting 519 bp *Eco*RI/*Xho*I fragment with the mutations was isolated by agarose gel electrophoresis. The human E3 expression vector pPROEX-1:E3 was digested with *Eco*RI and *Xho*I to remove the corresponding normal *Eco*RI/*Xho*I fragment. The resulting human E3 expression vector lacking the normal *Eco*RI/*Xho*I fragment was isolated by agarose gel electrophoresis. The isolated vector was ligated with the previously isolated *Eco*RI/*Xho*I fragment, resulting in the construction of the mutant expression vector pPROEX-1:E3(N-473->L). This process also removed the pre-existing *Bam*HI site. The removal of the *Bam*HI 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 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
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.

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.

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