Characterization of Human Dihydrolipoamide Dehydrogenase Mutant with Significantly Decreased Catalytic Power

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Dihydrolipoamide dehydrogenase (E3) (dihydrolipoamide: NAD⁺ oxidoreductase; EC 1.8.1.4) is an essential component in three α-keto acid dehydrogenase complexes (pyruvate, α-ketoglutarate and branched-chain α-keto acid dehydrogenase complexes) and the glycine cleavage system. E3 facilitates the reoxidation of the dihydrolipoyl prosthetic group of the acyltransferase components of the three α-keto acid dehydrogenase complexes and of the hydrogen-carrier protein of the glycine cleavage system. The decrease of the E3 activity can affect the activities of all three complexes, because E3 is commonly present in them. This results in increased urinary excretion of α-keto acids, elevated blood lactate, pyruvate, and branched chain amino acids. Patients with an E3 deficiency normally die young, because it is a critical genetic defect that affects parts of the central nervous system, such as the brain, which leads to serious diseases, including Leigh syndrome with permanent lactic acidaemia and mental retardation.

E3 exists as a homodimeric form containing one FAD as a prosthetic group at each subunit (Fig. 1). Each subunit consists of 474 amino acids with a molecular mass of 50,216 daltons. Along with glutathione reductase, thioredoxin reductase, mercuric reductase and trypanothione reductase, E3 belongs to the pyridine nucleotide-disulfide oxidoreductase family. The catalytic mechanisms of pyridine nucleotide-disulfide oxidoreductases are similar. All of these oxidoreductases have homodimeric structures containing an active disulfide center and a FAD in their each subunit. Through the FAD and active disulfide center, the oxidoreductases catalyze electron transfers between pyridine nucleotides (NAD⁺ or NADPH) and their specific substrates. The oxidoreductases consist of four structural domains (FAD, NAD, central and interface domains).

Knowledge about the binding modes of FAD and NAD⁺ to human E3 can be inferred from their X-ray crystallographic structure. On the other hand, the binding mode of dihydrolipoamide is not well known, because the structure of human E3 with dihydrolipoamide has not been determined yet. His-329 is located at the end of the presumed dihydrolipoamide binding channel (Fig. 1). This suggests that His-329 may be involved in interactions with dihydrolipoamide. Fig. 2 shows the sequence alignment of the His-329 region of human E3 with the corresponding regions of E3s from a range of sources, such as pigs, yeast, Escherichia coli and Pseudomonas fluorescens. His-329 is absolutely conserved in the various E3s including human E3, suggesting that it might be important for their structure and function. His-329 is a part of the long α-helix 8, which is composed of 16 amino acids and is a component of the central domain. His-329 is also located near FAD and the active disulfide center between Cys-45 and Cys-50, which are essential to the catalytic activity of human E3 (Fig. 3).
EXPERIMENTAL SECTION

Materials

*E. coli* XL1-Blue containing the human E3 expression vector, pPROEX-1:E3, was a generous gift from Dr. Mulchand S. Patel of the University at Buffalo, the State University of New York. The electrophoresis reagents, imidazole, iminodiacetic acid sepharose 6B, NAD$^+$, and NADH were obtained from Sigma-Aldrich (St. Louis, USA). Dihydrolipoamide was synthesized by the reduction of lipoamide using sodium borohydride. Isopropyl-β-D-thiogalactopyranoside (IPTG) was obtained from Promega (Madison, USA). Ni-NTA His-Bind Resin was obtained from Qiagen (Hilden, Germany). The primers and dNTPs were obtained from Bioline (Daejeon, Korea). The Mutagenesis Kit was from iNtRON Biotechnology (Seongnam, Korea).

Site-directed mutagenesis

Site-directed mutagenesis was carried out using a mutagenesis kit. Two mutagenic primers were used for the mutations. Primer A (5'-GGTCCAATGCTGGCTGCAAAAGCA-GAGGATGAAG-3': the mismatched bases are underlined) is an anti-sense oligomer with point mutations to convert His-329 (CAC) to Ala (GCC). Primer B (5'-CTTCATCCTCT-GCTTTGGCAGCCACGCAATGGACC-3': the mismatched bases are underlined) is the corresponding sense oligomer of the primer A. PCRs were carried out using the human E3 expression vector pPROEX-1:E3 as a template in a programmable PCR machine. Whole DNA sequence of human E3 coding region was sequenced to verify the integrity of DNA sequences other than the anticipated mutations.

Expression and purification of the human E3 mutant

3 ml of an overnight culture of *E. coli* DH5α containing the human E3 mutant expression vector were used to inoculate 1 L 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. 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 50 mM potassium phosphate buffer (pH 8.0) containing 100 mM NaCl and 20 mM imidazole (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 Ni-NTA His-Bind Resin column. The column had been washed with 2 column volumes of distilled water and then equilibrated with 5 column volumes of Binding buffer. After 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 50 mM imidazole. The E3 mutant was eluted with Binding buffer containing 250 mM imidazole.

SDS-polyacrylamide electrophoresis

SDS-PAGE analysis of proteins was performed in 12% SDS-PAGE gel. The gel was stained with Coomassie blue after electrophoresis.

Figure 2. Sequence alignment of the His-329 region of human E3 with the corresponding regions of E3s from a range of sources (from top to bottom; human, pig, yeast, *Escherichia coli* and *Pseudomonas fluorescens*). The UniProtKB ID and amino acid sequence from residue-319 to residue-337 are shown. H-329 and the corresponding residues are underlined. The alignment analysis was performed using the MAFFT program on the ExPASy Proteomics Server (Swiss Institute of Bioinformatics).

Figure 3. Location of His-329 in human E3. The two subunits of human E3 are shown as cartoons, with each secondary structure portrayed in a single color (red and green, respectively). FAD (yellow) and NAD$^+$ (blue) are shown as sticks and the active disulfide center (violet), Ala-451' (grey), His-452' (orange) and His-329 (cyan) are shown as spheres. His-329 is located close to FAD and the active disulfide center and can form van der Waals interactions with Ala-451' and His-452' from the other subunit. The structure was drawn using the PyMOL program (DeLano Scientific LLC). The PDB ID for the human E3 structure is 1ZMC.
E3 assay
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. 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).

Fluorescence spectroscopic study
The 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
To examine the role and importance of His-329 in the structure and function of human E3, it was site-specifically mutated to Ala as described in Experimental Section. The mutant was expressed in E. coli by IPTG induction (1 mM). The purification of the mutants was performed using a nickel affinity column. The purification steps were followed by SDS-polyacrylamide gel electrophoresis (Fig. 4). The gel showed that the mutants were 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. The kinetic experiments were performed in triplicate. The data was analyzed using the SigmaPlot Enzyme Kinetics Module, which generated double reciprocal plots (Fig. 5). The plots showed parallel lines, indicating that the mutant also catalyzes the reaction via a ping pong mechanism. The kinetic mechanism of human E3 is a ping-pong mechanism. The enzyme binds first to dihydrolipoamide and electrons are then transferred from dihydrolipoamide to the enzyme. This results in a two-electron reduced form of...
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Table 1. Steady state kinetic parameters of mutant and wild-type human E3s. The E3 assay was performed at 37°C in a 50 mM potassium phosphate buffer (pH 8.0) containing 1.5 mM EDTA. Values are mean ± S.D. of three independent determinations.

<table>
<thead>
<tr>
<th>Human E3</th>
<th>(k_{cat}) (s(^{-1}))</th>
<th>(K_m) toward dihydrolipoamide (mM)</th>
<th>(K_m) toward NAD(^+) (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>899±114</td>
<td>0.64±0.06</td>
<td>0.19±0.02</td>
</tr>
<tr>
<td>H329A mutant</td>
<td>37±0</td>
<td>0.29±0.01</td>
<td>0.16±0.00</td>
</tr>
</tbody>
</table>

The enzyme expels the oxidized lipoamide and binds to NAD\(^+\). The electrons are transferred from the enzyme to NAD\(^+\) to produce NADH, which will finally fall off the enzyme.

Figure 6. Fluorescence spectra of the wild-type (dotted line) and mutant (solid line) recombinant human E3s. The 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.

The mutation of His-329 to Ala would cause structural changes in human E3, which would induce alterations in the fluorescence properties and kinetic parameters of the mutant.
indicates that the FRET from Trp to FAD is significantly disturbed, implying that structural changes occur in the mutant. In conclusion, these findings indicate that the conservation of His-329 in human E3 is very important for the efficient catalytic activity and structure of the enzyme.

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REFERENCES