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Protein Engineering of an Artificial Intersubunit Disulfide Bond Linkage in Human Dihydrolipoamide Dehydrogenase

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Dihydrolipoamide dehydrogenase (E3) belongs to the protein family of pyridine nucleotide-disulfide oxidoreductases, including glutathione reductase (GR). The two subunits of human GR are covalently linked by an intersubunit disulfide bond between the pair of the Cys-90 residues. The corresponding residue (Ser-79) in human E3 was substituted to Cys using site-directed mutagenesis. The mutant was expressed in *Escherichia coli* and highly purified using an affinity column. About 40% of the mutants formed a spontaneous intersubunit disulfide bond linkage. This result implies that Ser-79 and possibly surrounding residues constitute one of the several intersubunit contact regions in human E3. It provides another good piece of evidence for the predicted high degree of the structural homology between human E3 and GR. Spectroscopic studies indicate conformational changes in the mutant.

Keywords: Dihydrolipoamide dehydrogenase, Disulfide bond linkage, α-Keto acid dehydrogenase complex, Site-directed mutagenesis, Structural homology.

Introduction

Dihydrolipoamide dehydrogenase (E3) (dihydrolipoamide: NAD^+ oxidoreductase; EC 1.8.1.4) is a homodimeric flavoprotein containing one FAD as a prosthetic group at each subunit. 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 in 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 is a member of the protein family of pyridine nucleotide-disulfide oxidoreductases. This family includes glutathione reductase (GR), thioredoxin reductase, and trypanothione reductase (William, 1976). Members of this family have homodimeric structures containing an active disulfide center and a FAD in each subunit. Through the FAD and active disulfide center, they catalyze electron transfers between pyridine nucleotides (NAD^+ or NADPH) and their specific substrates. The reactions are composed of two half-reactions, which are separated by a characteristic two-electron reduced state of the enzyme intermediate.

From the comparison studies of the structures of E3s and GRs from several sources, it becomes possible to predict the amino acid residues important to 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 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 shows similar properties to those of purified mammalian E3s (Kim *et al.*, 1991). 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; Liu *et al.*, 1995).

The three-dimensional structure of human GR has been extensively studied and well characterized (Thieme *et al.*, 1981; Pai and Schultz, 1983). One characteristic of human GR is the covalent linkage by a disulfide bond between the pair of Cys-90 residues in the two subunits. Cys-90 residue is located in one of the several intersubunit contact regions in human GR. Since the corresponding residue in human E3 is Ser-79, the intersubunit disulfide covalent linkage is absent in human E3. However, a significant number of

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other amino acid residues near Ser-79 have similar characteristics to those near Cys-90 in human GR, suggesting that this region in human E3 could also be an intersubunit contact region. In order to examine this suggestion, Ser-79 was replaced by Cys using site-directed mutagenesis. About 40% of the mutants formed a spontaneous intersubunit disulfide bond linkage and the rest of the mutants could form the disulfide bond linkage with the help of oxidant (H_2O_2), confirming the suggestion.

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, pROEX-1:E3, was a generous gift from Dr. Mulchand S. Patel of the 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 (Seoul, Korea).

Site-directed mutagenesis and construction of the human E3 mutant expression vector, pROEX-1:E3 (S-79->C) Strategies for the site-directed mutagenesis and construction of the mutant expression vector are shown in Fig. 1. Two polymerase chain reactions (PCR) were carried out using the primer pairs A with B and C with D, respectively. Primer A (5′-TCCCAACGACCGA AAACCTG-3′) and Primer D (5′-GCCAAAACAAGCCAAGC TTGG-3′) are sense and anti-sense oligomers, respectively, which can be used to amplify the whole E3 gene sequence. Primer B (5′-GCGAACTTCGCACATTTCAATTCC**CCTAGA**TGC-3′: the mismatched bases are underlined and the mutated *Xba*I site is written in bold letters) is an anti-sense oligomer with point mutations to convert Ser-79 to Cys and to destroy the *Xba*I site. Primer C (5′-GCA**TCTAGA**GGGAATTGAAATGTGCGAAG TTCGC-3′: the mismatched bases are underlined and the mutated *Xba*I site is written in bold letters) is the corresponding sense oligomer of the primer B.

The reactions were performed using the human E3 expression vector pROEX-1:E3 as a template with Vent polymerase in a programmable PCR machine. After a 2-min incubation at 95°C, thirty three 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 7-min incubation at 72°C was followed. The PCR with primers A and B produced an expected 281 bp fragment AB (Fig. 1) while the PCR with primers C and D generated an expected 1253 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 3-min incubation at 95°C, five rounds of temperature cycling were performed at 95°C for 30 sec, 50°C for 1 min, and 72°C for 90 sec. Thirty rounds of temperature cycling were then followed at 95°C for 30 sec, 43°C for 1 min, 72°C for 90 sec and a final 7-min incubation at 72°C was carried out. This PCR generated an expected 1501 bp fragment AD (Fig. 1) which contained the E3 sequence with the point mutations.

The fragment AD was digested with *Nar*I and *Eco*RI and then the *Nar*I/*Eco*RI fragment (909 bp) was isolated by agarose gel electrophoresis. The human E3 expression vector pROEX-1:E3 was digested with *Nar*I and *Eco*RI to remove the corresponding normal *Nar*I/*Eco*RI fragment. The vector lacking the *Nar*I/*Eco*RI sequence was ligated with the previously isolated *Nar*I/*Eco*RI fragment containing the mutations (Fig. 1). The ligation resulted in the construction of the mutant expression vector, pROEX-1:E3(S-79→C).

Expression and purification of the human E3 mutant An overnight culture of *E. coli* XL1-Blue (1 ml) containing pROEX-1:E3(S-79→C) 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. 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 (binding buffer) and then recollected by centrifugation at 4000 × *g* for 5 min. They were resuspended in 10 ml of Binding buffer, lysed by a sonication treatment, and centrifuged at 10,000 × *g* for 20 min.

Since the expression vector, pROEX-1, was designed to insert six histidines into the amino-terminus of any expressed proteins, the mutant could be purified using immobilized metal affinity chromatography. The nickel iminodiacetic acid sepharose 6B column was prepared by loading an iminodiacetic acid sepharose 6B resin into a column of 5 ml volume with 10 cm height. The column was washed with 5 column vol of distilled water and then with the same volume of 50 mM $NiSO_4$ solution. The column had been washed with 2 column vol of distilled water and then equilibrated with 5 column vol of binding buffer.

For the purification of the E3 mutant, the supernatant was loaded on the column. The column was washed with 10 column vol of binding buffer followed by the same volume of binding buffer containing 150 mM imidazole. The E3 mutant was eluted with binding buffer containing 500 mM imidazole.

SDS-polyacrylamide gel electrophoresis and gel density estimation The samples were taken from each purification step and incubated for 5 min at 95°C with sample buffer containing or lacking β-mercaptoethanol. They were analyzed by SDS-polyacrylamide gel electrophoresis and the gel was stained with Coomassie Blue. The gel density was estimated using an imaging densitometer (Bio-Rad Laboratories, Hercules, USA).

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 (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.

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). Samples were excited at 296 nm and the emissions were 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, USA).

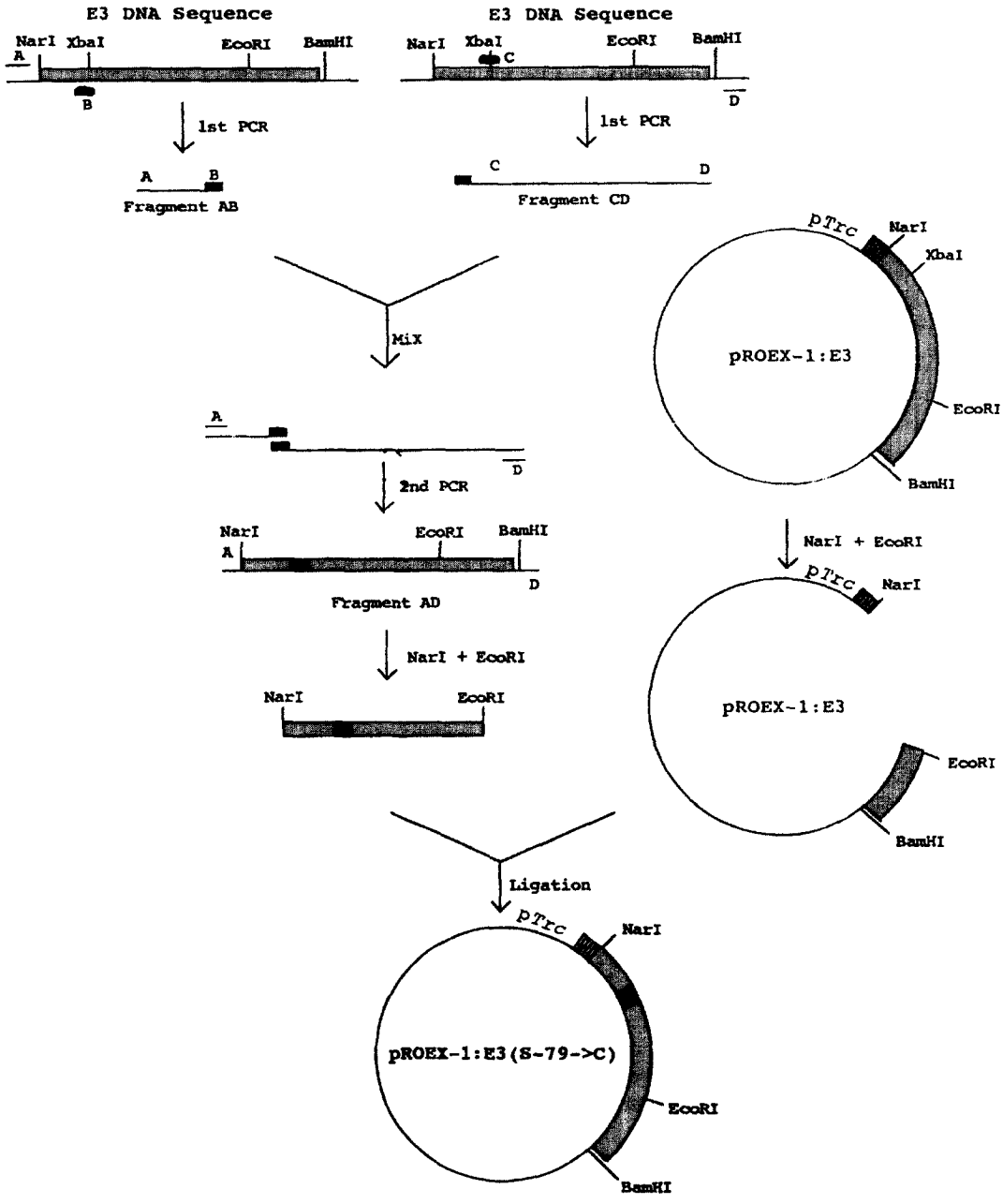

Fig. 1. Strategies for PCR and the construction of the human E3 mutant expression vector pROEX-1:E3(S-79→C). ▬, E3 coding region; ■, mutagenic primer region; ▥, His-tag region.

Results and Discussion

Table 1 shows an amino acid sequence comparison around Ser-79 in human E3 and corresponding residues from other E3s and human GR. The two subunits of human GR are covalently linked through an intersubunit disulfide bond between their Cys-90 residues. However, the two subunits of human E3 are bound to each other only through noncovalent interactions (Williams, 1976). This may be mainly due to the fact that human E3 has a Ser residue instead of the Cys residue. Cys-90 residue belongs to one of the several intersubunit contact regions in human GR. A significant number of other amino acid residues near Ser-79 in human E3 have similar characteristics to those near Cys-90 in human GR (Jentoft *et al.*, 1992). This suggests that this region in human E3 might also be one of the several intersubunit contact regions (Jentoft *et al.*, 1992). It may be possible to introduce an artificial intersubunit

Table 1. Comparison of amino acid sequences around the Ser residue from E3s and human GR. Ser-79 in human E3 and the corresponding residues in other E3s and human GR are highlighted by italic bold letters.

Protein	Source	Amino acid sequence
E3	*E. coli*	71-H G I V F ***G*** E P K T D-81
E3	Yeast	72-R G I D V ***N*** G D I K I-82
E3	Pig	74-R G I E M ***S*** E V R L N-84
E3	Human	74-R G I E M ***S*** E V R L N-84
GR	Human	85-Y G F P S ***C*** E G K F N-95

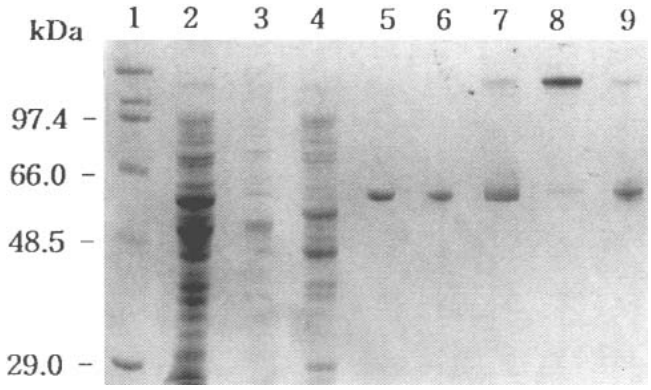

Fig. 2. SDS-polyacrylamide gel electrophoresis for the human E3 mutant collected from each purification step. SDS-PAGE was carried out according to Laemmli (1970). Protein bands were visualized by staining with Coomassie-blue. Lane 1, molecular weight marker (from bottom to top, bovine erythrocytes carbonic anhydrase, porcine heart fumarase, bovine serum albumin, rabbit muscle phosphorylase b); lane 2, supernatant; lane 3, elution with binding buffer; lane 4, elution with binding buffer containing 150 mM imidazole; lane 5, elution with binding buffer containing 500 mM imidazole; lane 6, previously purified recombinant human E3 as a control; lane 7, same as lane 5 except that the sample buffer did not contain β-mercaptoethanol; lane 8, same as lane 7 except that the E3 mutant was incubated with 0.1% H_2O_2 for 5 min before treatment with the sample buffer; lane 9, same as lane 8 except that the normal recombinant human E3 was used.

disulfide bond linkage in human E3 by replacing Ser-79 with Cys.

In order to examine this possibility, Ser-79 in human E3 was substituted to Cys using site-directed mutagenesis. Screening of the mutant expression vector, pROEX-1:E3 (S-79→C), was performed by *Xba*I digestion. There is a unique *Xba*I site in the normal E3 cDNA sequence. One adenine base of the *Xba*I sequence was simultaneously replaced by guanine during the site-directed mutagenesis. The substitution of TCTAGA to TCTAGG resulted in the destruction of the *Xba*I site without any changes to the primary structure. The simultaneous *Xba*I and *Bam*HI digestion of the mutant expression vector produced only one DNA band on agarose gel (data not shown) due to the destruction of the *Xba*I site, while the same treatment of the E3 expression vector generated two DNA bands including a 1209 bp *Xba*I-*Bam*HI fragment.

The samples collected at each purification step were analyzed by SDS-polyacrylamide gel electrophoresis (Fig. 2). After equilibration with binding buffer, the supernatant solution (lane 2) was applied to the column. The *E. coli* proteins were washed out with binding buffer (lane 3) and then with binding buffer containing 150 mM imidazole (lane 4). The mutant was eluted with binding buffer containing 500 mM (lane 5). The purified mutant was dialyzed against 10 mM potassium phosphate buffer (pH 7.5) containing 0.5 mM EDTA.

To examine whether the mutant could form an intersubunit disulfide bond, the purified mutant was treated with a sample buffer lacking a reductant (β-mercaptoethanol) and then analyzed on the SDS-gel. As shown in Fig. 2, lane 7, about 40% of the mutant could form an intersubunit disulfide bond, so that they appeared on the gel as a dimeric molecule with a higher molecular weight. Somehow, in addition to one main protein band, two minor bands were observed on the gel. Their estimated molecular weights were higher than the expected value of the dimer, probably due to the intersubunit disulfide bond, which might slow down the migration of the denatured mutants on the SDS-polyacrylamide gel. With treatment of 0.1% H_2O_2 as an oxidant, the rest of the mutants could form the disulfide bond (lane 8) while most of the normal enzyme could not form the intersubunit disulfide bond even after treatment with the oxidant (lane 9). These results indicate that the Ser-79 residue, like Cys-90 residue in human GR, is located in one of the several intersubunit contact regions in human E3. The two Ser-79 residues are close enough to each other for the partial spontaneous intersubunit disulfide bond formation. However, they are not close enough for the full disulfide bond formation. The help of the oxidant was needed for the full disulfide bond formation.

The E3 activity of the mutant was determined to be 1203 unit/mg at substrate concentrations of 2 mM dihydrolipoamide and 3 mM NAD^+. This value is about 2-fold higher than that of the normal enzyme.

Due to FAD, E3 has characteristic UV-visible absorption spectrum patterns of flavoproteins from 320 nm to 550 nm. The spectrum of E3 has two peaks at 455 nm and around 365 nm (Fig. 3, dotted line). 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 proteins may result in these characteristic features in the spectrum. To detect any structural changes caused by the artificial disulfide bond, the UV-visible spectra of the mutant and normal E3s were recorded as described in *Materials and Methods*. As shown in Fig. 3, the UV-visible spectrum of the mutant (solid line) is

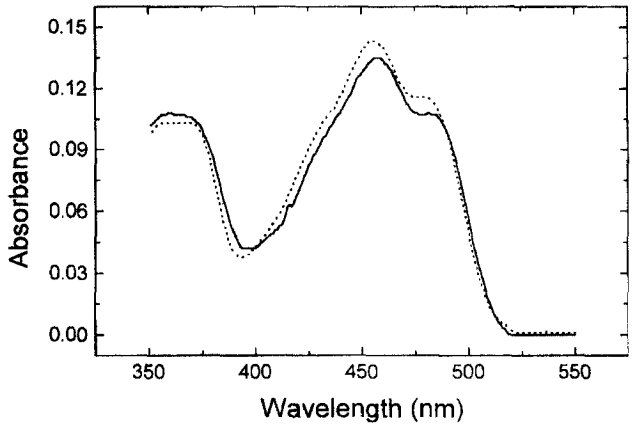

Fig. 3. UV-visible spectra of the mutant (11.9 μM, solid line) and wild-type (12.5 μM, dotted line) recombinant human E3s. The spectra were recorded using a Shimazu UV 160A spectrophotometer and the data from 350 nm to 550 nm were transferred to an ASCII file and the spectra drawn using the MicroCal Origin program.

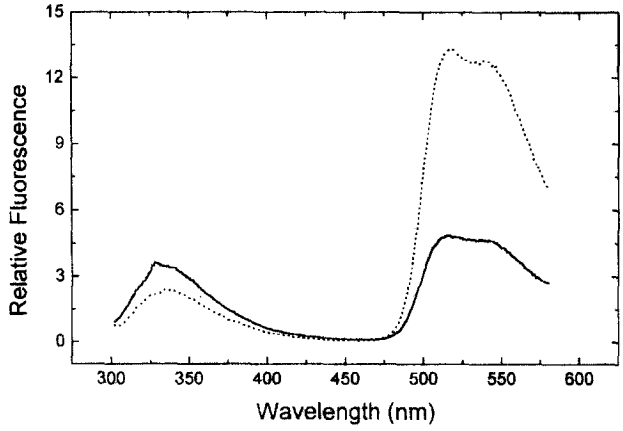

Fig. 4. Fluorescence spectra of the mutant (4.8 μM, solid line) and wild-type (12.5 μM, dotted line) recombinant human E3s. Enzymes were excited at 296 nm and emissions were observed from 302 nm to 580 nm. The data were transferred to an ASCII file and the spectra drawn using the MicroCal Origin program.

similar to that of the normal enzyme except that its 455 nm peak and the unique shoulder was slightly shifted to the right by about 3–10 nm, indicating that some conformational changes may have occurred. The ratio between the maximum absorbance value around 458 nm due to FAD and that around 274 nm due to aromatic amino acid residues are good indicators for E3 purity. The ratio 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). Therefore, fluorescence spectroscopy was used for the structure-function study of human E3. When human E3s were excited at 296 nm, two fluorescence emissions were observed in both mutant and normal enzymes, as shown in Fig. 4. The first emission was from 310 nm to 370 nm with a maximum emission around 336 nm and the second emission was from 480 nm to over 580 nm with a maximum emission around 519 nm.

The intensities of the two fluorescence emissions of the mutant and normal E3s are quite different. The ratio between maximum emissions of the first and the second fluorescences of the mutant was 1.5 while that of the normal enzyme was 5.5. This result clearly confirmed the previously indicated structural changes in the mutant. The structural changes caused by the substitution and the intersubunit disulfide bond linkage in the mutant could result in the observed differences in the fluorescence spectra.

Both E3 and GR belong to the protein family of pyridine nucleotide-disulfide oxidoreductases and it is well known that E3 and GR have good structural homology. Since the three-dimensional structure of human GR has been extensively studied and is well known (Thieme *et al.*, 1981; Pai and Schultz, 1983), then based on this, the structural comparisons between E3s and GRs have been tried (Carothers *et al.*, 1989; Thekkumkara *et al.*, 1989) and a structural model for human E3 has been proposed (Jentoft *et al.*, 1992). Three-dimensional structures of *Azotobacter vinelandii* E3 (Schierbeek *et al.*, 1989; Mattevi *et al.*, 1991) and *Pseudomonas putida* E3 (Mattevi *et al.*, 1992) have been reported. They show a high degree of structural homology to human GR. However, significant conformational differences have also been observed. The crystal structure of mammalian E3 is not yet known. It can be assumed that mammalian E3 also has a high degree of structural homology to human GR. However, even though there are 33% sequence identities between the two enzymes, it is inappropriate to pre-conclude the assumption. In this research, Ser-79 of human E3, the corresponding residue of the intersubunit disulfide bond linkage (Cys-90) in human GR, was substituted to a Cys residue. The mutant could form the expected intersubunit disulfide linkage. This successful introduction of the artificial intersubunit disulfide bond in human E3, based on the prediction from the structural comparison of human E3 with human GR, clearly shows that, at least, Ser-79 and surrounding residues constitute one of the several intersubunit contact regions in human E3. It also provides another good piece of evidence for the predicted high degree of structural homology between human E3 and GR.

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