Autodegradation of Protein Disulfide Isomerase

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Protein disulfide isomerase (PDI) and its degradation products were found in HepG2, COS-1, and CHO-K1 cells. Whether or not the products were formed through autodegradation of PDI was examined, since PDI contains the CGHC motif, which is the active center of proteolytic activity in ER-60 protease. Commercial bovine PDI was autodegraded to produce a trimmed PDI. In addition, human recombinant PDI also had autodegradation activity. Mutant recombinant PDIs with CGHC motifs of which cysteine residues were replaced with serine or alanine residues were prepared. However, they were not autodegraded, suggesting the cysteine residues of motifs are necessary for autodegradation.

Key words: protein disulfide isomerase; endoplasmic reticulum; proteolytic activity

Protein disulfide isomerase (PDI) was first shown to form or rearrange the disulfide bonds of newly synthesized polypeptides in the endoplasmic reticulum (ER),¹–⁵ and is now known to be a multifunctional protein with chaperone, antichaperone,⁶–⁸ transglutaminase,⁷ and ATPase activities.⁹ Among these functions, disulfide isomerase activity was shown to be catalyzed by the two CGHC motifs present in the PDI molecule.¹⁰ However, CGHC motifs are also found in ER-60 protease¹¹–¹³ and ERp72.¹⁴,¹⁵ Using a mutated recombinant human ER-60 protease, the activity was found to be due to the C-terminal cysteine residue of the CGHC motif.¹⁶ However, there has been no report on the proteolytic activity of PDI. It has been reported that a trimmed PDI, which was produced by removing a few-kDa fragment from PDI, was present in a PDI preparation.¹⁷ This suggests the possibility that PDI was autodegraded through its potential proteolytic activity. Hence, we examined autodegradation of PDI.

To analyze PDI in animal cells, HepG2, COS-1, and CHO-K1 cells, which were grown in Dulbecco’s modified Eagle’s medium or α-modified minimum essential medium containing 10% fetal bovine serum, were rinsed twice with phosphate-buffered saline and then treated with 10% trichloroacetic acid at 4°C for 30 min on the growth plates to inactivate proteolytic enzymes. The treated cells were collected and dissolved in 7.2 M urea/1.6% Triton X-100/0.8% DTT/2% lithium dodecyl sulfate at 25°C. Samples were electrophoresed on SDS-polyacrylamide gel and analyzed by Western blotting using anti-PDI antiserum as described previously.¹⁸ In each cell type, PDI and a smaller protein which had lost approximately 5 kDa of the intact PDI fragment were detected (Fig. 1, lanes 2, 4 and 6). No band was detectable on the immunoblot probed with pre-immune rabbit serum (Fig. 1, lanes 1, 3 and 5). Therefore, this trimmed protein was assumed to be a degradation product of PDI. However, it was unclear whether the degradation was caused by some protease or PDI itself, which contains CGHC motifs in its molecule like the ER-60 protease does.

Since the commercial bovine liver PDI preparation (Takara Shuozo, Kyoto), which was purified by the method of Lambert and Freedman,¹⁹ contained a contaminating 20-kDa protein, it was further purified by hydroxyapatite column chromatography as described previously.²⁰ The purified PDI, which was free from the 20-kDa protein, was incubated in 10 mM bis(2-hydroxyethyl) iminotris (hydroxymethyl) methane (bis-Tris)/HCl buffer, pH 5.9, at 37°C. Time-dependent production of a trimmed PDI, which was approximately 5 kDa smaller than PDI, was observed (Fig. 2, lanes 1–4). The mobility of the trimmed PDI was the same as that of the trimmed PDI found in animal cells. The N-terminal sequence of the trimmed PDI was APDEEXHVLV. This

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Abbreviations: PDI, protein disulfide isomerase; ER, endoplasmic reticulum; bis-Tris, bis(2-hydroxyethyl) iminotris (hydroxymethyl) methane; β-ME, β-mercaptoethanol; IPTG, isopropyl-1-thio-β-D-galactopyranoside; PMSF, phenylmethylsulfonylfluoride; KPB, potassium phosphate buffer; PAGE, polyacrylamide gel electrophoresis; CBB, Coomassie Brilliant Blue R-250

Fig. 1. PDI and Its Degradation Products in Animal Cells. HepG2 (lanes 1 and 2), COS-1 (lanes 3 and 4) and CHO-K1 cells (lanes 5 and 6), which were grown on 60-mm plates, were treated with 10% trichloroacetic acid and then immunoblotted with pre-immune serum (lanes 1, 3, and 5) or anti-PDI antiserum (lanes 2, 4, and 6) as described in the text.
Fig. 2. Autodegradation of Bovine PDI.

PDI was incubated in 10 mM bis-Tris buffer, pH 5.9, for the indicated times at 37°C with (lanes 1-4) or without 100 mM β-ME (lane 5). Samples were put on SDS-PAGE (10% gel). Proteins were stained with Coomassie Brilliant Blue R-250 (CBB).

is the same as the N-terminal sequence of intact bovine PDI. Hence, the protein was assumed to be cleaved on the C-terminal side of PDI. The reaction required β-mercaptoethanol (β-ME) (Fig. 2, lane 5). However, PDI could not degrade bovine serum albumin under the assay conditions (data not shown).

Human recombinant PDI was prepared and purified as follows. cDNA of PDI (ATCC number 59478: pHTB) was obtained from the American Type Cell Culture Collection. The nucleotide fragment, encoding the amino acid sequence of PDI except for the N-terminal signal peptide region, was amplified by polymerase chain reaction, and then subcloned into the expression vector pET-20b(+) (Novagen, WI) between the Nde I and Hind III sites. The sequence of the PDI construct in the expression vector was confirmed by the method of Sanger et al., the following differences being observed compared with the published sequence. The sequence of nucleotides 1153-1161 was found to be CTGCCGGA and not CTTGCCGA. The corresponding amino acid changes are Arg343, Ala344, and Gly345, which were predicted previously by other researchers, against Leu343, Phe344, and Gla345. Thus, probably the original nucleotide sequence in this region may have been an error.

The expression plasmid was used to transform the E. coli host strain, BL21(DE3) (Novagen, WI), grown as 2-L cultures in the presence of 500 μg/ml carbenicillin at 37°C, and induced with 0.4 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) for 2 h at 30°C. The recombinant protein, which had the same mobility as that of PDI in Chang liver cells (human hepatocytes) on SDS-PAGE, was expressed in a water-soluble form (Fig. 3a, lanes 4 and 5). The cells were collected by centrifugation, disrupted by sonication in 50 ml of 20 mM HEPES buffer, pH 6.8, containing 5 mM EDTA, 1 mM phenylmethylosulfonylfluoride (PMSF), and 10% glycerol (buffer A), and then centrifuged at 5,000 × g for

Fig. 3. Expression, Purification, and Autodegradation of Recombinant Human PDI.

Panel a: Expression of human cDNA was done as described in the text. The proteins were electrophoresed on a 10% gel, and then stained with CBB (lanes 1 and 2) or immunoblotted with anti-PDI serum (lanes 3-5). Lanes 1-4, total proteins of BL21(DE3) cells carrying plasmids for the expression of recombinant wild type PDI; lane 5, total proteins of Chang liver cells (2 μg of protein). Induction of the recombinant proteins was done by incubation with 0.4 mM IPTG for 2 h (lanes 2 and 4). Panel b: Purification of the recombinant wild type PDI was done as described in the text. An extract of BL21(DE3) cells carrying an expression plasmid (lane 1), the supernatant fraction (lane 2) and pellet fraction (lane 3) obtained on centrifugation, the fraction eluted from a heparin column with 175 mM KCl (lane 4), and the combined fractions containing PDI obtained on TSK gel G3000SW column chromatography (lane 5), and HTP column chromatography (lane 6), respectively, were electrophoresed on a 10% gel. The proteins were stained with CBB. Panel c: The recombinant wild-type PDI was incubated at 37°C for 0 (lane 1) or 1 h (lane 2). Samples were run on SDS-PAGE (10% gel). Proteins were immunoblotted with anti-PDI serum.
10 min at 4°C. The supernatant was put on AF-heparin Toyopearl 650M column (Tosoh, Tokyo) chromatography, with equilibration with buffer A. The column was washed with buffer A until the absorbance reached the baseline, and then eluted stepwise with buffer A containing 50 and 175 mM KCl. The effluence obtained with 175 mM KCl was put on a TSK gel G3000SW column (Tosoh, Tokyo) equilibrated with 20 mM Tris-HCl, pH 7.4, containing 0.15 M NaCl, 5 mM EDTA, 1 mM PMSF, and 10% glycerol. The peak fractions of the recombinant PDI were collected and then dialyzed overnight against 10 mM potassium phosphate buffer (KPB), pH 7.4, containing 0.5 mM PMSF. The dialysate was put on a HTP-cartridge (Bio Rad Laboratories, Cal.) equilibrated with 25 mM KPB. The column was washed with 25 mM KPB. The recombinant PDI was eluted with 75 mM KPB. The purified PDI preparation thus obtained gave a single band on SDS-polyacrylamide gel electrophoresis (PAGE) (Fig. 3b, lane 6). Then, the purified recombinant PDI was incubated under the same conditions as described above for bovine PDI. Autodegradation of the recombinant PDI was also observed (Fig. 3c). From the results described above, it seems likely that the trimmed PDI observed in vivo is the product of autodegradation of PDI. However, it cannot be excluded that limited proteolysis of PDI was caused by some protease existing in the ER.

Next, we prepared mutant recombinant PDIs with site-directed mutations in the CGHC motifs. The expression plasmid for the wild type PDI was used as the template for site-directed mutagenesis. To change Cys-36, Cys-39, Cys-380, and Cys-383, which are four cysteine residues in two CGHC motifs, to serine or alanine, polymerase chain reactions were done with mutagenic primers by the method of Ito et al.23) The amplified PCR fragments were cloned into pET-20b (+) at the Nde I and Hind III sites and expressed in BL21(DE3) (Fig. 4). All mutants, Cys-36 and Cys-380 both modified to serine (C36S/C380S), Cys-39 and Cys-383 both modified to serine or alanine (C39S/C383S or C39A/C383A), Cys-36 and Cys-39 both modified to serine (C36S/C39S), and Cys-380 and Cys-383 both modified to serine (C380S/C383S), were confirmed by nucleotide sequence analysis. The mutant recombinant PDIs were purified by the same procedures as those for wild type recombinant PDI. The purified mutant recombinant PDIs were incubated under the conditions described above. However, they were not autodegraded (data not shown). This suggests that the cysteine residues in CGHC motifs are required for autodegradation of PDI. The degradation mechanism remains unknown. This will be studied in the near future.

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References


