Expression and Site-directed Mutagenesis of Human Protein Disulfide Isomerase in Escherichia coli

THIS MULTIFUNCTIONAL POLYPEPTIDE HAS TWO INDEPENDENTLY ACTING CATALYTIC SITES FOR THE ISOMERASE ACTIVITY*

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Protein disulfide isomerase (PDI, EC 5.3.4.1) is a highly unusual multifunctional polypeptide, being identical to the β subunit of prolyl 4-hydroxylase, a cellular thyroid hormone binding protein and a component of the microsomal triglyceride transfer protein complex, and highly similar to a polypeptide acting in vitro as a glycosylation site binding protein. It has two -Cys-Gly-His-Cys- sequences which, it has been proposed, act as catalytic sites for the isomerase activity, but few data have been available to indicate whether one or both of them do indeed act as catalytic sites and whether the two presumed catalytic sites act independently or cooperatively. We report here on the expression of human PDI in Escherichia coli with three different signal sequences. All three polypeptide variants were secreted into the periplasmic space as fully active enzymes. Oligonucleotide-directed mutagenesis was used to convert either one or both of the -Cys-Gly-His-Cys- sequences to -Ser-Gly-His-Cys-. The PDI activity of both polypeptides containing a single modified sequence was about 50% of that of the wild-type polypeptide, whereas the polypeptide with two modified sequences had no isomerase activity. It is thus concluded that both -Cys-Gly-His-Cys- sequences act as catalytic sites for the isomerase activity, and the two catalytic sites appear to operate independently of one another.

Protein disulfide isomerase (PDI, EC 5.3.4.1) catalyzes protein disulfide formation, reduction, or isomerization in vitro, depending on the reaction conditions, and is regarded as the in vivo catalyst for disulfide bond formation in the synthesis of secretory proteins (1–3). Cloning and nucleotide sequencing were first reported for the rat enzyme (4). Surprisingly, PDI was subsequently found to be identical to the β subunit of prolyl 4-hydroxylase, an αβtetramer (5, 6), a cellular thyroid hormone binding protein (7, 8), and a component of the microsomal triglyceride transfer protein complex (9), and highly similar to a polypeptide acting in vitro as a glycosylation site binding protein (10–12). Complete DNA-derived amino acid sequences are now available for this multifunctional polypeptide from several sources (for reviews see Refs. 13–15).

The human PDI polypeptide consists of 491 amino acid residues and a signal sequence of 17 additional amino acids (5). The polypeptide has two thioredoxin-like regions, each containing a -Cys-Gly-His-Cys- sequence, which, it has been proposed (2, 4), act as catalytic sites for the isomerase activity. Some observations support this proposal (2), but it is also possible that only one of the two -Cys-Gly-His-Cys- sequences is involved in the catalysis (16). It is furthermore unknown whether the two presumptive catalytic sites act independently or whether the polypeptide is folded in such a manner that the two -Cys-Gly-His-Cys- sequences come close to each other and act cooperatively (2, 17).

We report here on expression of human PDI in Escherichia coli. Three signal sequences were tested in order to obtain secretion of an active enzyme into the periplasmic space. Site-directed mutagenesis of the two -Cys-Gly-His-Cys- sequences was used to determine whether both of these sequences act as catalytic sites for the isomerase activity and whether they act independently or cooperatively.

**MATERIALS AND METHODS**

Unless otherwise indicated, the DNA manipulations were performed as described in Sambrook et al. (18). The oligonucleotides were synthesized in an Applied Biosystems DNA synthesizer (Department of Biochemistry, University of Oulu), and the mutations were created using an oligonucleotide-directed in vitro mutagenesis system (Amersham Corp.).

Construction of the Expression Plasmids pKK233-PDI, pKK233-PDI/ompA'Ag, and pTM2-PDI—To construct pKK233-PDI (Fig. 1A), a 1903-bp EcoRI-HindIII fragment of a full-length cDNA clone for human PDI (5) was ligated to a similarly digested M13mpl8 (3). Because of the internal NcoI site in the PDI cDNA, first the NcoI***-NcoI fragment of the PDI insert of pKK233-2 was ligated in a 1:1:1 molar ratio.

The original signal sequence for human PDI was replaced by the slightly modified signal sequence of the E. coli ompA protein (19) (Fig. 1B). A 1968-bp BamHI-HindIII fragment from a PDI cDNA clone, a 58-bp Ncol***-BamHI fragment prepared from four oligonucleotides encoding the signal sequence, and an Ncol-HindIII-digested vector pKK233-2 were ligated in a 1:1:1 molar ratio.

The vector pTM2-2 was a gift from Dr. Tiliang Deng (University of California at San Diego, La Jolla, CA) and is a slight modification of pTO-N (20). When constructing pTM2-PDI (Fig. 1A), an Ncol restriction site (Ncol*** in Fig. 1A) was created at the end of the signal sequence of PDI as described above for pKK233-PDI, thus changing the codon for Ala (the extreme C-terminal amino acid of the original signal sequence) into that for Met. First the Ncol-HindIII fragment and then the Ncol*'-Ncol fragment of the PDI insert of

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‡ The abbreviations used are: PDI, protein disulfide isomerase; bp, base pair(s); IPTG, isopropyl-β-D-thiogalactopyranoside; ompA, outer membrane protein A of E. coli; ompT, outer membrane protein T of E. coli; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.
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**Fig. 1. Construction of plasmids pKK233-PDI, pKK233-PDI/ompAsig, and pTM2-PDI.** A shows the maps of the plasmids constructed. The PDI cDNA is a black arc in each vector. *Ptrc*, trc promoter; *T7* promoter; AMP', ampicillin resistance gene. B shows the signal sequences of the plasmids. *Arrow* indicates the differing amino acids between the signal sequence of pKK233-PDI and the original PDI and pKK233-PDI/ompAsig and the authentic *E. coli* ompA protein, respectively. The first amino acid of the mature PDI polypeptide is indicated by +1.

**Fig. 2. SDS-PAGE and Western blot analysis of the expression of PDI in *E. coli*.** Lanes 1–5 show Coomassie staining: lane 1, purified chick prolyl 4-hydroxylase; lane 2, purified human prolyl 4-hydroxylase; lane 3, purified human PDI; lane 4, purified human PDI (arrow) of which is identical to PDI; lane 5, Triton X-100 soluble proteins of *E. coli* subunit (arrow) of which is identical to PDI), with the same samples as in lanes 2–5; lanes 6 and 11, Triton X-100 soluble proteins of JM105 cells carrying pKK233-PDI or BL21 (DE3) (IacIq); lanes 6–10, Western blots: lanes 6–9, the same samples as in lanes 2–5; lanes 10 and 11, Triton X-100 soluble proteins of BL21 (DE3) (IacIq) cells carrying pKK233-PDI or BL21 (DE3) (IacIq) cells carrying pTM2-PDI. Molecular size standards in kDa are given on the right.

This mutated construct was ligated to the Ncol-HindIII site of the vector pTM2-2, as when constructing pKK233-PDI.

**Construction of the Expression Plasmids pTM2-PDI, pTM2-PDI+, and pTM2-PDI++—The codon for the first Cys in both presumed catalytic sites of PDI (6) was changed to the codon for Ser by site-directed mutagenesis using M13 mp19 containing either the PDI cDNA clone *β*-210 (coding for the N-terminal catalytic site) or *β*-202 (coding for the C-terminal catalytic site) as a template (5). When constructing pTM2-PDI, (see Fig. 3 in "Results"), the Ncol***-Ncol fragment of the PDI cDNA clone in M13 (see above) was cloned to the Nterminal altered catalytic site from the mutated M13 *β*-202 vector. The radioactivity of the immunoprecipitate was quantified from the bands of the filter by densitometry from Coomassie staining of the SDS-PAGE of fractionated Triton X-100 soluble and periplasmic proteins using a Kortes K495000 densitometer.

N-terminal sequences were determined in an Applied Biosystems model 477A with on-line 120A liquid-pulsed Sequencer. The products of the PDI activity were compared with those of the purified PDI from the periplasmic fraction of JM105 cells carrying pKK233-PDI or pKK233-PDI/ompAsig. The radioactivity of the immunoprecipitate was quantified by liquid scintillation counting and compared with the radioactivity obtained from the total cell extracts in order to determine the proportion of PDI. Samples of immunoprecipitates were also subjected to 10% SDS-PAGE and treated for fluorography. The purity of the immunoprecipitable counts represented the PDI polypeptide, as fluorography indicated that only minor contaminant protein bands were present (not shown).

The expression level obtained with pTM2-PDI was estimated by densitometry from Coomassie staining of the SDS-PAGE of fractionated Triton X-100 soluble and periplasmic proteins using a Kortes K495000 densitometer.

**RESULTS AND DISCUSSION**

Expression of Human PDI in *E. coli*—Three expression plasmids varying in their signal sequences were constructed.
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Levels of expression of human PDI with the different expression plasmids in various E. coli strains

The experimental details are given under "Materials and Methods."

| Expression plasmid       | E. coli strain                  | Triton X-100 soluble protein | Periplasmic protein |
|--------------------------|--------------------------------|------------------------------|---------------------|
| pKK233-PDI, pKR233-PDI/ompAsig | JM105, SF100, SF110, BL21 (DE3) (lacIq) | 0.5–1                        | 2–4                |
| pTM2-PDI                 | BL21 (DE3)                      | 0.4–0.8                      | 1.5–3.5             |

|avage protein was detected in experiments with the parental plasmids pKK233-2 or pTM2-2 (Fig. 2). The expression level was 0.5–1% of the Triton X-100 soluble protein with both pKK233-PDI and pTM2-PDI/ompAsig and 10–15% with pTM2-PDI (Table I).

Several experiments using the JM105 cells nevertheless gave a 43-kDa band instead of the expected 55-kDa band, and both bands were present in some experiments (Fig. 2). Little or no 43-kDa form was seen in experiments with E. coli strains SF100, SF110, and BL21 (DE3) (lacIq) which are deficient in the ompT protease (26).

Secretion of Human PDI into the Periplasmic Space as a Mature, Fully Active Polypeptide—E. coli cells carrying pKK233-PDI, pKK233-PDI/ompAsig, or pTM2-PDI were grown and treated by the osmotic shock method to release their periplasmic proteins. These were then studied by SDS-PAGE followed by Western blotting. A 55-kDa band was seen with all three plasmids (Fig. 2). Quantification experiments using [35S]methionine labeling or Coomassie staining followed by densitometry indicated that PDI represented 2–4% of the periplasmic protein when using pKK233-PDI or pKK233-PDI/ompAsig and 35–40% when using pTM2-PDI (Table I). After the osmotic shock only a small fraction of the PDI was still cell-associated, and >95% was found in the supernatant. The high expression level obtained with pTM2-PDI and almost 100% secretion into the periplasmic space makes this system suitable for large-scale production of PDI, e.g. for crystallographic work. When using the standard purification protocol for PDI, 1 ml of bacterial cell cultures harboring pTM2-PDI will yield about 30 μg of pure PDI.

Determination of the N-terminal amino acid sequences of the expressed polypeptides containing either the authentic PDI signal peptide (coded by pKK233-PDI) or the ompA signal peptide (coded by pTM2-PDI) demonstrated that both variants of the signal sequences were correctly cleaved (not shown).

PDI activity was determined in the periplasmic and total Triton X-100 soluble protein. After correction of the values for the proportion of PDI, the specific activity of PDI expressed in E. coli strains SF100, SF110, and BL21 (DE3) (lacIq) which are deficient in the ompT protease (26).

Construction of the Mutant Expression Plasmids pTM2-PDI<sub>ab</sub>, pTM2-PDI<sub>a</sub>, and pTM2-PDI<sub>ab</sub>—To study whether the two -Cys-Gly-Cys- sequences represent catalytic sites for PDI activity, three modified plasmids were constructed from pTM2-PDI. In pTM2-PDI, the N-terminal presumptive catalytic site was modified by replacing the codon for its first Cys by a codon for Ser (Fig. 3). In pTM2-PDI<sub>a</sub> the corresponding change was made to the sequence coding for the first Cys in the presumed C-terminal catalytic site, while in pTM2-PDI<sub>ab</sub>...
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PDI activity of wild-type and mutated PDI polypeptides in the periplasmic or total Triton X-100 soluble protein fraction of E. coli BL21 (DE3) cells harboring different expression plasmids

| Plasmid    | Relative PDI activity | RNase assay* | Insulin assay* |
|------------|-----------------------|--------------|---------------|
| pTM2-PDI   |                       | V_{max}      | V_{max}       |
| pTM2-PDIa  | 100                   | 100          | 100           |
| pTM2-PDIb  | 46 ± 9                | 40-60        | 55            |
| pTM2-PDlb  | 47 ± 10               | 40-60        | 51            |
| pTM2-PDlb  | 2 ± 2                 | 0            | 0             |

* Catalsysis of the rate of regeneration of incorrectly disulfide-linked RNase to native form (22). Values for standard conditions are the mean ± S.D. from six experiments; the V_{max} was determined in one experiment and is given as a range of possible values due to the inaccuracy of the assay.

The experimental details are given under "Materials and Methods."