The acidic C-terminal domain of protein disulfide isomerase is not critical for the enzyme subunit function or for the chaperone or disulfide isomerase activities of the polypeptide

Peppi Koivunen, Annamari Pirneskoski, Päivi Karvonen, Johanna Ljung¹, Tarja Helaakoski, Holger Notbohm² and Kari I. Kivirikko³

Collagen Research Unit, Biocenter Oulu and Department of Medical Biochemistry, University of Oulu, Kajaanintie 52A, FIN-90220 Oulu, Finland, ¹Department of Medical Biochemistry and Biophysics, Karolinska Institutet, S-17177 Stockholm, Sweden and ²Institute for Medical Molecular Biology, Medical University of Lübeck, D-23538 Lübeck, Germany

³Corresponding author
e-mail: KKivirik@lbiok-1.oulu.fi

Protein disulfide isomerase (PDI) is a multifunctional polypeptide that acts as a subunit in the animal prolyl 4-hydroxylases and the microsomal triglyceride transfer protein, and as a chaperone that binds various peptides and assists their folding. We report here that deletion of PDI sequences corresponding to the entire C-terminal domain *c*, previously thought to be critical for chaperone activity, had no inhibitory effect on the assembly of recombinant prolyl 4-hydroxylase in insect cells or on the *in vitro* chaperone activity or disulfide isomerase activity of purified PDI. However, partially overlapping critical regions for all these functions were identified at the C-terminal end of the preceding thioredoxin-like domain *a'*. Point mutations introduced into this region identified several residues as critical for prolyl 4-hydroxylase assembly. Circular dichroism spectra of three mutants suggested that two of these mutations may have caused only local alterations, whereas one of them may have led to more extensive structural changes. The critical region identified here corresponds to the C-terminal α helix of domain *a'*, but this is not the only critical region for any of these functions.

Keywords: chaperone/collagen/microsomal triglyceride transfer protein/prolyl 4-hydroxylase/protein disulfide isomerase

Introduction

Protein disulfide isomerase (PDI, EC 5.3.4.1), a major protein within the lumen of the endoplasmic reticulum (ER), catalyzes disulfide bond formation in the biosynthesis of proteins translocated into this cell compartment (for reviews, see Noiva and Lennarz, 1992; Freedman *et al.*, 1994). It is a modular protein that consists of domains *a*, *b*, *b'*, *a'* and *c* (Edman *et al.*, 1985; Pihlajaniemi *et al.*, 1987; Kemmink *et al.*, 1995). The existence of an additional domain *e*, thought to be located between domains *a* and *b*, is now very unlikely (Kemmink *et al.*, 1995; Darby *et al.*, 1996). The *a* and *a'* domains are similar to thioredoxin (Edman *et al.*, 1985), and both

contain the sequence -CGHC-, representing two independently acting catalytic sites for PDI activity (Hawkins and Freedman, 1991; Vuori *et al.*, 1992a,c; LaMantia and Lennarz, 1993; Lyles and Gilbert, 1994; Darby and Creighton, 1995). Nuclear magnetic resonance (NMR) characterization of human domain *a* produced in *Escherichia coli* indicates that it also has the thioredoxin fold (Kemmink *et al.*, 1995, 1996). Recent NMR studies on domain *b* and preliminary data on domain *b'* suggest that these likewise may have the thioredoxin fold, even though they have no catalytic site sequences and show no significant amino acid sequence similarity to thioredoxin (Kemmink *et al.*, 1997). The main part of the PDI polypeptide may thus consist of two catalytically active thioredoxin modules and two inactive ones (Kemmink *et al.*, 1997).

The C-terminal domain *c*, which represents a putative Ca^{2+} -binding region (Freedman *et al.*, 1994), is rich in acidic amino acids; 18 out of the 29 extreme C-terminal residues in the human polypeptide being either aspartate or glutamate (Pihlajaniemi *et al.*, 1987). Its C-terminus contains the -KDEL motif, which is both necessary and sufficient for the retention of a polypeptide within the lumen of the ER (Pelham, 1990).

The PDI polypeptide has a number of additional functions. It serves as the β subunit of the animal prolyl 4-hydroxylases (Pihlajaniemi *et al.*, 1987; Veijola *et al.*, 1994; Helaakoski *et al.*, 1995) and of the microsomal triglyceride transfer protein (Wetterau *et al.*, 1990), and as a chaperone-like polypeptide that binds various peptides within the lumen of the ER and probably assists in folding of many newly synthesized proteins (LaMantia and Lennarz, 1993; Cai *et al.*, 1994; Otsu *et al.*, 1994; Puig and Gilbert, 1994; Puig *et al.*, 1994; Rupp *et al.*, 1994; Hayano *et al.*, 1995; Yao *et al.*, 1997). PDI may also have other, less well characterized functions (Freedman *et al.*, 1994; Prockop and Kivirikko, 1995; Kivirikko and Myllyharju, 1998; Kivirikko and Pihlajaniemi, 1998).

One major function of the PDI polypeptide in the prolyl 4-hydroxylases (Vuori *et al.*, 1992b; John *et al.*, 1993; Veijola *et al.*, 1994; Helaakoski *et al.*, 1995) and the microsomal triglyceride transfer protein (Wetterau *et al.*, 1991; Ricci *et al.*, 1995; Lamberg *et al.*, 1996) is to keep their highly insoluble α subunits in a catalytically active, non-aggregated conformation. This function is similar to that of some molecular chaperones such as Hsp90 in other proteins (Freedman *et al.*, 1994) and may thus be related to the chaperone function of the polypeptide. Photoaffinity labelling studies with a radioactive tripeptide probe have localized a peptide-binding site in rat PDI to a single 26 amino acid tryptic peptide that begins at the end of domain *a'* and includes most of domain *c* (Noiva *et al.*, 1993). Binding of this photoaffinity-labelled peptide to PDI

Fig. 1. Schematic representation of the C-terminal region of human PDI. The amino acid sequence is shown in one-letter codes and the corresponding numbering is indicated below. The black bars show the various deletions introduced in the polypeptide, while the point mutations are shown above the amino acid sequence. The predicted protein secondary structure elements present are indicated by a white bar (α helix) and a jagged line (β sheet), whereas the unmarked region between them is likely to form a loop.

inhibits the chaperone activity of the latter (Puig *et al.*, 1994).

The present work sets out to study whether domain *c* and the C-terminal end of domain *a'* are critical for the prolyl 4-hydroxylase subunit function and the chaperone and disulfide isomerase activities of the PDI polypeptide. Our data indicate that deletion of the entire domain *c* has no inhibitory effect on any of these functions, whereas the extreme C-terminal α helix of domain *a'* appears to constitute one critical region for all these functions.

Results

Effect of deletions in the C-terminal region of PDI on assembly of the human type I prolyl 4-hydroxylase tetramer

Prolyl 4-hydroxylase from vertebrates is an $\alpha_2\beta_2$ tetramer (Kivirikko *et al.*, 1989, 1992) in which PDI functions as the β subunit (Pihlajaniemi *et al.*, 1987). The vertebrate α subunit has two isoforms, $\alpha(I)$ and $\alpha(II)$, which contribute to the $[\alpha(I)]_2\beta_2$, type I, and $[\alpha(II)]_2\beta_2$, type II, tetramers (Helaakoski *et al.*, 1995). The photoaffinity-labelled 26 amino acid tryptic peptide of rat PDI (Noiva *et al.*, 1993) corresponds to a 27 amino acid sequence, residues 452–478, in the 491 amino acid human PDI polypeptide (Figure 1). Secondary structure predictions and modelling based on the structures of thioredoxin (see Martin, 1995) and domain *a* of PDI (Kemink *et al.*, 1995, 1996) suggest that the first five residues in this sequence correspond to the end of the last α helix of domain *a'*, while most of the residues correspond to those of domain *c* (Figure 1).

The 27 amino acid sequence was deleted from human PDI ($\Delta 452$ –478) and the mutant polypeptide was expressed together with the wild-type human $\alpha(I)$ subunit in insect cells. The cells were harvested 72 h after infection, homogenized in a buffer containing Triton X-100 and centrifuged. The Triton X-100-soluble proteins were then studied by non-denaturing PAGE followed by Coomassie staining (Figure 2A) or by Western blotting with a polyclonal antibody to human PDI (Figure 2B), and assayed for prolyl 4-hydroxylase activity by a procedure based on the hydroxylation-coupled decarboxylation of 2-oxo[1- ^{14}C]glutarate (Table I). The Triton X-100-soluble proteins from all the experiments described below were also studied by SDS-PAGE under reducing conditions to verify the expression levels and sizes of the various mutant

polypeptides. None of the mutations reported in the present study was found to influence the expression level (details not shown).

Deletion of the 27 amino acid sequence from PDI was found to prevent prolyl 4-hydroxylase assembly, as no band corresponding to the enzyme tetramer was seen in non-denaturing PAGE (Figure 2A and B, lanes 2), and no prolyl 4-hydroxylase activity was generated in insect cells (Table I). Deletion of the N-terminal half of this sequence, residues 452–465 (Figure 1), likewise prevented tetramer assembly virtually entirely, as no band corresponding to the tetramer was seen in non-denaturing PAGE (Figure 2A and B, lanes 3), and only a very minor amount of prolyl 4-hydroxylase activity was generated in insect cells (Table I). In contrast, deletion of the C-terminal half, residues 466–478, had no inhibitory effect (Figure 2A and B, lanes 4; Table I). Region 452–465 subsequently was studied by means of four further deletions. A three residue deletion, $\Delta 452$ –454, involving an FLE triplet (Figure 1), totally prevented tetramer assembly (Figure 2A and B, lanes 5), whereas deletion of residues 455–457 (SGG), 458–461 (QDGA) or 462–465 (GDDD) had no inhibitory effect (Figure 2A and B, lanes 6–8; Table I). Thus the only critical region within the 27 residue sequence was its N-terminal FLE triplet.

Additional deletion experiments indicated that the critical region when studied by means of small deletions extended in the N-terminal direction from the 27 amino acid sequence, as the deletions 449–451 (FKK), 446–448 (LDG), 446–449 (LDGF), 444–449 (RTLDGF), 440–443 (YNGE) and 436–439 (TVID) also prevented tetramer assembly (Figure 2A and B, lanes 9–14; Table I). In contrast, additional deletions in the N-terminal direction, 432–435 (SADR) and 431–433 (ASA), did not prevent tetramer assembly (Figure 2A and B, lanes 15 and 16, and Table I), except that the enzyme tetramer formed with the $\Delta 432$ –435 (SADR) deletion mutant had an abnormal mobility (Figure 2B, lane 15) and could not be detected in the Coomassie-stained gel (Figure 2A, lane 15), and the amount of enzyme activity generated in the insect cells was only 60% of that obtained with the wild-type PDI (Table I).

The role of domain *c* was studied further by deleting residues 479–487 (Figure 1) or by creating a translation termination codon after residue 461, thus deleting the 30 extreme C-terminal residues of the polypeptide. Neither of these deletions inhibited assembly of an active prolyl

Fig. 2. Non-denaturing PAGE analysis of prolyl 4-hydroxylase tetramer formation from the wild-type human prolyl 4-hydroxylase $\alpha(I)$ subunit and the wild-type or various deletion mutants of human PDI. The samples were extracted with a buffer containing 0.1% Triton X-100 and analysed by non-denaturing 8% PAGE followed by Coomassie staining in (A) or Western blotting with a polyclonal antibody to human PDI in (B). The sample in lane 18 was run separately. T indicates the migration of tetramers and PDIs those of the deletion mutant PDI polypeptides. Lanes 1–18 show Triton X-100-soluble proteins from cells co-infected with the human $\alpha(I)$ virus together with viruses PDI-wt (1), PDI Δ 452–478 (2), PDI Δ 452–465 (3), PDI Δ 466–478 (4), PDI Δ 452–454 (5), PDI Δ 455–457 (6), PDI Δ 458–461 (7), PDI Δ 462–465 (8), PDI Δ 449–451 (9), PDI Δ 446–448 (10), PDI Δ 446–449 (11), PDI Δ 444–449 (12), PDI Δ 440–443 (13), PDI Δ 436–439 (14), PDI Δ 432–435 (15), PDI Δ 431–433 (16), PDI Δ 479–487 (17) and PDI Δ 462–491 (18).

4-hydroxylase tetramer (Figure 2A and B, lanes 17 and 18; Table I).

Effect of deletions in the C-terminal region of PDI on assembly of the hybrid *C.elegans* α subunit–human PDI prolyl 4-hydroxylase dimer and the human type II enzyme tetramer

The α subunit of prolyl 4-hydroxylase from *Caenorhabditis elegans* forms an active enzyme with both the *C.elegans* and human PDI polypeptides in insect cells, but the prolyl 4-hydroxylases containing the *C.elegans* α subunit differ from vertebrate enzymes in being $\alpha\beta$ dimers (Veijola *et al.*, 1994, 1996). The role of the C-terminal region of the human PDI polypeptide in this dimer assembly was studied by expressing all the mutant polypeptides described above in insect cells together with the *C.elegans* α subunit. Dimer assembly was assessed by non-denaturing PAGE of the Triton X-100-soluble proteins followed by Western blotting with a polyclonal antibody to human PDI. The data obtained were identical to those with the human $\alpha(I)$ subunit in that the only critical region among the amino acids studied covered residues 436–454 (Figure 3). Additional experiments demonstrated that the critical region among the residues studied for assembly of the human type II prolyl 4-hydroxylase [$\alpha(II)$] $_2\beta_2$ tetramer was identical (details not shown).

Effect of point mutations in the C-terminal α helix of domain a'

Thirteen residues in the C-terminal region of domain a' were converted individually to other amino acids (Figure 1). Nine of them were within the predicted extreme C-terminal α helix of domain a' , while four residues were in the preceding region predicted to form a loop structure.

Table I. Prolyl 4-hydroxylase activity of Triton X-100-soluble proteins from insect cells expressing the $\alpha(I)$ subunit of human prolyl 4-hydroxylase together with either the wild-type PDI or its deleted forms

Polypeptides expressed	Prolyl 4-hydroxylase activity (d.p.m./10 μ g)	%
$\alpha(I)$ + PDI-wt	4510 $\pm$ 910	100
$\alpha(I)$ + PDI Δ 452–478	<90	<2**
$\alpha(I)$ + PDI Δ 452–465	210 $\pm$ 290	5**
$\alpha(I)$ + PDI Δ 466–478	4480 $\pm$ 750	99
$\alpha(I)$ + PDI Δ 462–465	3950 $\pm$ 1130	88
$\alpha(I)$ + PDI Δ 458–461	5370 $\pm$ 800	119
$\alpha(I)$ + PDI Δ 455–457	5990 $\pm$ 1590	133
$\alpha(I)$ + PDI Δ 452–454	<90	<2**
$\alpha(I)$ + PDI Δ 449–451	<90	<2**
$\alpha(I)$ + PDI Δ 446–449	<90	<2**
$\alpha(I)$ + PDI Δ 446–448	<90	<2**
$\alpha(I)$ + PDI Δ 444–449	<90	<2**
$\alpha(I)$ + PDI Δ 440–443	<90	<2**
$\alpha(I)$ + PDI Δ 436–439	<90	<2**
$\alpha(I)$ + PDI Δ 432–435	2710 $\pm$ 730	60*
$\alpha(I)$ + PDI Δ 431–433	4130 $\pm$ 260	92
$\alpha(I)$ + PDI Δ 479–487	4420 $\pm$ 860	98
$\alpha(I)$ + PDI Δ 462–491	5180 $\pm$ 650	125

Values are given in d.p.m./10 μ g of extractable cell protein (mean $\pm$ SD for three experiments) and as percentages of the value obtained with the wild-type PDI (PDI-wt). The amount of prolyl 4-hydroxylase activity generated by association of the human $\alpha(I)$ subunit with the endogenous insect cell PDI, as determined in each experiment by expressing the $\alpha(I)$ subunit alone, was subtracted from all the values. This enzyme activity level was typically ~3–5% of that obtained by co-expression of the $\alpha(I)$ subunit with the wild-type PDI. Significances of the differences relative to PDI-wt are indicated by asterisks (* p < 0.05; ** p < 0.001).

One of these residues, F449, was converted to five different amino acids (Figure 1). The mutant PDI polypeptides were then expressed together with the wild-type human

Fig. 3. Non-denaturing PAGE analysis of prolyl 4-hydroxylase dimer formation from the wild-type *C.elegans* prolyl 4-hydroxylase α subunit and the wild-type or mutant human PDI polypeptides. The samples were extracted as in Figure 2 and analysed by 8% PAGE followed by Western blotting with a polyclonal antibody to human PDI. D indicates the migration of dimers and PDIs those of the deletion mutant PDI polypeptides. Lanes 1–17 show the 0.1% Triton X-100-soluble proteins from cells co-infected with the *C.elegans* α virus together with viruses human PDI-wt (1), PDI Δ 452–478 (2), PDI Δ 452–465 (3), PDI Δ 466–478 (4), PDI Δ 452–454 (5), PDI Δ 455–457 (6), PDI Δ 458–461 (7), PDI Δ 462–465 (8), PDI Δ 449–451 (9), PDI Δ 446–448 (10), PDI Δ 446–449 (11), PDI Δ 444–449 (12), PDI Δ 440–443 (13), PDI Δ 436–439 (14), PDI Δ 432–435 (15), PDI Δ 431–433 (16) and PDI Δ 479–487 (17).

Fig. 4. Analysis of prolyl 4-hydroxylase tetramer formation from human α (I) and mutant PDI/ β subunits expressed in insect cells. The Triton X-100-soluble samples were electrophoresed on non-denaturing 8% PAGE and analysed by Western blotting with a polyclonal antibody to the human PDI polypeptide. The samples were run on two separate gels. T indicates the migration of tetramers and PDI that of the mutant PDI/ β subunits. Lanes 1–17 show samples from co-expression of human α (I) together with PDI-wt (1), PDI (V437D) (2), PDI (I438E) (3), PDI (D439A) (4), PDI (R444A) (5), PDI (L446E) (6), PDI (D447A) (7), PDI (G448R) (8), PDI (F449R) (9), PDI (F449Y) (10), PDI (F449E) (11), PDI (F449W) (12), PDI (K450A) (13), PDI (K451A) (14), PDI (F452R) (15), PDI (L453E) (16) and PDI (E454A) (17).

α (I) subunit in insect cells, and tetramer assembly was studied by non-denaturing PAGE analysis followed by Western blotting and prolyl 4-hydroxylase activity assays as above.

In the initial experiments, three mutations were found to either markedly reduce (R444A and L453E) or totally prevent (F449R) prolyl 4-hydroxylase assembly (Figure 4, lanes 5, 16 and 9, respectively). These mutations also caused a concomitant decrease in or loss of the amount of prolyl 4-hydroxylase activity generated in the cells to 43% (R444A), 13% (L453E) or 0% (F449R) of that obtained with the wild-type PDI (Table II). Mutations G448R and K451A also significantly decreased the amount of prolyl 4-hydroxylase activity (Table II), whereas mutation of any of the eight other residues gave no significant decrease in tetramer assembly (Figure 4) or in the amount of prolyl 4-hydroxylase activity, D439A giving a statistically significant minor increase in the amount of activity (Table II).

The role of F449 was studied further by individual mutations to glutamate, tyrosine, tryptophan and alanine. F449E inhibited tetramer assembly essentially just as completely as F449R, in that no tetramer could be detected in the non-denaturing PAGE (Figure 4, lane 11) and the amount of prolyl 4-hydroxylase activity generated in insect cells was only 9% of that obtained with the wild-type PDI (Table II). F449W and F449A also reduced the amount of prolyl 4-hydroxylase activity generated in the insect cells to 59 and 63%, respectively, lower values than those obtained with any of the mutants involving amino acids other than R444, F449 or L453, whereas F449Y reduced the activity only to 79% (Table II).

Effect of deletions in the C-terminal region of PDI on the chaperone activity of the polypeptide *in vitro*

To study the effect of C-terminal deletions on the chaperone activity of PDI, selected mutants were expressed in insect cells and the recombinant polypeptides purified by a procedure consisting of gel filtration and anion exchange chromatography. The purified polypeptides were then assayed for chaperone activity by measuring the reactivation of denatured rhodanese, a protein containing no disulfide bonds (Song and Wang, 1995). Measurements were made at three concentrations of the wild-type or mutant PDI polypeptides, the data obtained at 10 μ M being shown in Table III.

Domain *c* appeared to have no role in the chaperone activity, as the rate of rhodanese reactivation obtained with PDI Δ 462–491 was almost identical to that obtained with the wild-type PDI (as shown for 10 μ M concentrations in Table III). The three amino acid deletion 458–461 on the N-terminal side of the 30 amino acid deletion likewise had no effect on the chaperone activity, whereas four deletions, 455–457 (SGG), 452–454 (FLE), 449–451 (FKK) and 446–448 (LDG), had a distinct inhibitory effect (Table III). As described above, three of these deletions also prevented prolyl 4-hydroxylase assembly, whereas the Δ 455–457 deletion had no inhibitory effect.

The critical region for the chaperone activity appeared to be shorter than for the subunit function, as the deletions 440–443 (YNGE) and 431–433 (ASA) gave no inhibition (Table III). The F449R mutation that totally prevented prolyl 4-hydroxylase assembly had no inhibitory effect on the chaperone activity (Table III).

Table II. Prolyl 4-hydroxylase activity of Triton X-100-soluble proteins from insect cells expressing the $\alpha(I)$ subunit of human prolyl 4-hydroxylase together with either the wild-type PDI or its point mutant forms

Polypeptides expressed	Prolyl 4-hydroxylase activity (d.p.m./10 μ g)	%
$\alpha(I)$ + PDI-wt	3490 $\pm$ 560	100
$\alpha(I)$ + PDI(V437D)	3560 $\pm$ 200	102
$\alpha(I)$ + PDI(I438E)	2770 $\pm$ 1170	79
$\alpha(I)$ + PDI(D439A)	4150 $\pm$ 110	119**
$\alpha(I)$ + PDI(R444A)	1500 $\pm$ 1140	43*
$\alpha(I)$ + PDI(L446E)	3750 $\pm$ 1760	107
$\alpha(I)$ + PDI(D447A)	3200 $\pm$ 860	92
$\alpha(I)$ + PDI(G448R)	2420 $\pm$ 300	69**
$\alpha(I)$ + PDI(F449R)	0 $\pm$ 0	0***
$\alpha(I)$ + PDI(F449E)	320 $\pm$ 170	9***
$\alpha(I)$ + PDI(F449Y)	2750 $\pm$ 380	79*
$\alpha(I)$ + PDI(F449W)	2050 $\pm$ 1340	59*
$\alpha(I)$ + PDI(F449A)	2200 $\pm$ 160	63**
$\alpha(I)$ + PDI(K450A)	3930 $\pm$ 280	113
$\alpha(I)$ + PDI(K451A)	2600 $\pm$ 530	74*
$\alpha(I)$ + PDI(F452R)	4030 $\pm$ 1440	115
$\alpha(I)$ + PDI(L453E)	470 $\pm$ 310	13***
$\alpha(I)$ + PDI(E454A)	2640 $\pm$ 1170	76

Values are given in d.p.m./10 μ g of extractable cell protein (mean $\pm$ SD for at least three experiments) and as percentages of the value obtained with the wild-type PDI (PDI-wt). The enzyme activity generated by expressing the $\alpha(I)$ subunit alone was subtracted from all the values. Significances of the differences relative to PDI-wt are indicated by asterisks (* p < 0.05; ** p < 0.01; *** p < 0.001).

Table III. Chaperone activity of purified wild-type PDI and its mutants

Polypeptide studied	Reactivation level of rhodanase % rhodanase reactivated	% PDI-wt
PDI-wt	35 $\pm$ 9	100
PDI Δ 462–491	32 $\pm$ 9	91
PDI Δ 458–461	30 $\pm$ 4	86
PDI Δ 455–457	13 $\pm$ 5	37**
PDI Δ 452–454	14 $\pm$ 4	40**
PDI Δ 449–451	11 $\pm$ 4	31**
PDI Δ 446–448	28 $\pm$ 4	80*
PDI Δ 440–443	41 $\pm$ 5	117
PDI Δ 431–433	36 $\pm$ 6	103
F449R	43 $\pm$ 12	123
Lysozyme	0	0
No polypeptide	2 $\pm$ 3	6**

Values are given as percentages of the activity obtained with native rhodanase (mean $\pm$ SD) and as percentages of the value obtained with wild-type PDI (PDI-wt) for at least three experiments. Significances of the differences relative to PDI-wt are indicated by asterisks (* p < 0.05; ** p < 0.001). All measurements were performed in the presence of 10 μ M PDI-wt or its mutant forms, or 10 μ M lysozyme.

Effect of deletions and point mutations in the C-terminal region on the disulfide isomerase and reductase activities of PDI

Nine PDI deletion mutants were expressed in *E. coli* in a form containing an N-terminal histidine affinity tag and purified by an affinity chromatography based on this tag. The purified polypeptides were then used to study their disulfide isomerase and reductase activities. The former activity was measured by studying oxidative refolding of a fully reduced RNase (Lyles and Gilbert, 1991) and the latter by an insulin disulfide reduction assay (Holmgren, 1979).

Table IV. Disulfide isomerase and reductase activities of purified PDI mutants expressed in *E. coli*

Polypeptide	Isomerase activity % PDI-wt	Reductase activity % PDI-wt
PDI-wt	100	100
PDI Δ 462–491	98 $\pm$ 22	86 $\pm$ 7*
PDI Δ 458–461	77 $\pm$ 7*	125 $\pm$ 2**
PDI Δ 455–457	67 $\pm$ 3**	114 $\pm$ 6
PDI Δ 452–454	17 $\pm$ 2***	49 $\pm$ 4**
PDI Δ 449–451	20 $\pm$ 8***	50 $\pm$ 1*
PDI Δ 446–448	19 $\pm$ 6***	47 $\pm$ 0**
PDI Δ 440–443	22 $\pm$ 1***	44 $\pm$ 3***
PDI Δ 436–439	24 $\pm$ 0***	43 $\pm$ 1**
PDI Δ 431–433	12 $\pm$ 1***	60 $\pm$ 3**
PDI (R444A)	83 $\pm$ 35	79 $\pm$ 1*
PDI (G448R)	85 $\pm$ 10**	93 $\pm$ 1
PDI (F449R)	21 $\pm$ 1***	43 $\pm$ 3**
PDI (F449Y)	42 $\pm$ 17***	87 $\pm$ 6
PDI (F449W)	133 $\pm$ 3*	94 $\pm$ 2
PDI (L453E)	23 $\pm$ 8***	48 $\pm$ 6*

Values are given as percentages of the activity obtained with PDI-wt, mean $\pm$ SD for at least two experiments. Significances of the differences relative to PDI-wt are indicated by asterisks (* p < 0.05; ** p < 0.01; *** p < 0.001). Isomerase activity and reductase activity were measured in the presence of 1 μ M PDI-wt or its mutant forms.

Domain *c* had no role in the disulfide isomerase or reductase activities of PDI, as the former activity of the PDI Δ 462–491 mutant was identical and the latter very similar to those of the wild-type PDI (Table IV). Deletions 458–461 (QDGA) and 455–457 (SGG), which caused no inhibition of prolyl 4-hydroxylase assembly, reduced the disulfide isomerase activity by ~20 and 30%, respectively, whereas the insulin reductase activity was not reduced (Table IV). The Δ 455–457 mutant was also purified from insect cells and found to have an isomerase activity that was 75% of that of the wild-type PDI (details not shown).

Deletions in the region 431–454 caused a major decrease in the disulfide isomerase activity of the polypeptide to ~20% (Table IV). These deletions include Δ 431–433 (ASA), which caused no inhibition of prolyl 4-hydroxylase tetramer assembly. All these deletions also lowered the insulin disulfide reductase activity, although to a lesser extent, by ~50% (Table IV). The Δ 452–454 mutant was also purified from insect cells and found to have an isomerase activity equal to 41% of that of the wild-type PDI (details not shown), while the polypeptide purified from *E. coli* had an activity level of only ~17% (Table IV). It is possible, therefore, that the deletions have a stronger effect on the properties of the polypeptides expressed in *E. coli* than in insect cells.

The effects of six of the 17 point mutations were also studied as above. Two of these, F449R and L453E, which inhibited prolyl 4-hydroxylase assembly completely or almost completely (Table II), also had major effects on the disulfide isomerase and reductase activities (Table IV). The R444A mutation, which inhibited prolyl 4-hydroxylase assembly by ~60% (Table II), had only a minor effect on the disulfide isomerase and the reductase activities (Table IV), as did G448R, which inhibited prolyl 4-hydroxylase assembly by ~30% (Table IV). Mutation F449Y, causing ~20% reduction in prolyl 4-hydroxylase activity, reduced disulfide isomerase activity by ~60% but had little if any effect on the reductase activity, whereas

Fig. 5. CD spectrum analysis of the purified wild-type PDI and its mutant forms in the far-UV region (190–260 nm). Wild-type PDI is indicated by a solid line, PDI Δ 452–478 by a dotted line, PDI Δ 449–451 by a dashed line and PDI F449R by a dotted and dashed line.

F449W had no inhibitory effect on either (Table IV), although it did reduce prolyl 4-hydroxylase assembly by ~40% (Table II).

Circular dichroism spectrum analysis of PDI and its mutant forms

To study whether the loss of subunit function, isomerase and/or chaperone activity in the case of some of the PDI mutants was due to an overall change in the secondary structure rather than a local effect, circular dichroism (CD) spectra in the far UV region were recorded for two deletion mutants of PDI and one point mutant. One of the two deletion mutants, Δ 449–451 (FKK), showed a major difference in its CD spectrum relative to the wild-type PDI, whereas the CD spectrum of the deletion mutant Δ 452–478 was essentially identical to that of the wild-type PDI (Figure 5). One preparation of F449R gave a spectrum which was identical to that of the wild-type PDI (details not shown), whereas another preparation gave a spectrum that was slightly different (Figure 5), possibly due to minor amounts of contaminants.

Discussion

It has been demonstrated previously that the two -CGHC-catalytic sites of PDI are not involved in either its subunit function or its chaperone activity. A double mutant polypeptide in which both catalytic sites had been inactivated by converting them to -SGHC- formed a fully active prolyl 4-hydroxylase tetramer (Vuori *et al.*, 1992c) and microsomal triglyceride transfer protein dimer as effectively as did the wild-type polypeptide (Lamberg *et al.*, 1996). Such a PDI mutant also accelerated the *in vitro* refolding of denatured rhodanese and the *in vivo* folding of human lysozyme in yeast (Hayano *et al.*, 1995). Similarly, PDI alkylated at the thiols of the two catalytic sites was as effective as the wild-type polypeptide in accelerating refolding *in vitro* of a protein containing no disulfide bonds (Quan *et al.*, 1995).

Photoaffinity-labelling experiments with a radioactive tripeptide probe have localized a peptide-binding site in rat PDI to a single tryptic peptide that corresponds to a 27 amino acid sequence in human PDI, beginning at the end of domain *a'* and being located mainly in the highly acidic domain *c* (Noiva *et al.*, 1993). The actual binding site has been hypothesized to consist of a groove formed by acidic residues present in domain *c* (Noiva *et al.*, 1997). Binding of this tripeptide to PDI markedly reduced the *in vitro* chaperone activity of the latter (Puig *et al.*, 1994), and a similar effect was also seen when a 28 amino acid peptide was added to an *in vitro* refolding system for a protein with no disulfide bonds (Quan *et al.*, 1995).

The present data indicate that domain *c* in fact has no significant role in any of the main functions of PDI, i.e. the subunit function, the chaperone activity and the disulfide isomerase activity, since the PDI mutant lacking residues 462–491 formed prolyl 4-hydroxylase as effectively as did the wild-type polypeptide and was fully active as a chaperone and as a disulfide isomerase. Thus the hypotheses concerning the roles of domain *c* (Noiva *et al.*, 1997) have to be revised. Nevertheless, a critical region for all these functions was found to be located at the end of domain *a'*, a region of which the C-terminus overlaps with the N-terminus of the previously identified peptide-binding region. Our data thus suggest that the tripeptide probe used previously (Noiva *et al.*, 1993; Puig *et al.*, 1994) may have become bound close to the N-terminal end of the 27 amino acid region rather than to the highly acidic sequence present in domain *c*.

Klappa *et al.* (1998) recently studied binding of peptides longer than the tripeptide used by Noiva *et al.* (1993) to PDI. Peptides of 10–15 amino acids could be cross-linked to linear combinations of various PDI domains which included domain *b'*; and even domain *b'* alone exhibited efficient binding (Klappa *et al.*, 1998). However, cross-linking experiments with 'scrambled' RNase A demonstrated that domain *b'* was not sufficient for an efficient binding of this longer peptide, and the additional presence of domain *a'*–*c* was essential (Klappa *et al.*, 1998). The domain *a'* construct was built to also contain domain *c*, and thus the data do not necessarily indicate that domain *c* played any role in binding. In agreement with these results, Darby *et al.* (1998) recently reported that the smallest PDI fragment showing an efficient catalysis of disulfide bond rearrangement was the *b'*–*a'*–*c* construct.

Secondary structure predictions of domain *a'* suggest that the critical region identified by us mainly corresponds to its extreme C-terminal α helix and the preceding loop structure. CD spectrum analyses suggested that the large $\Delta 452$ –478 deletion and the F449R point mutation may have caused only local alterations, while the $\Delta 449$ –451 (FKK) deletion may have led to more extensive structural changes. However, the data do not exclude the possibility that the mutations may have produced only limited changes in the secondary structure in the region around the site of the mutation, but this may have led to wider effects on tertiary structure such as interactions between domain *a'* and other domains. The large effects of many mutations on the disulfide isomerase activity might be due to the latter explanation. Nevertheless, it has been reported previously that binding of the tripeptide probe to PDI, presumably between residues 452 and 461 based on our data and those of Noiva *et al.* (1993), at 93% saturation decreased the activity of PDI in the folding of reduced, denatured bovine pancreatic trypsin inhibitor to 21% and of reduced, denatured RNase to 43% (Puig *et al.*, 1994). These values are not very different from those found by us with many deletions and point mutations, and may thus be explained by local changes. The -CGHC- sequences present in domains *a* and *a'* represent two independently acting catalytic sites for the isomerase activity (Hawkins and Freedman, 1991; Vuori *et al.*, 1992a,c; LaMantia and Lennarz, 1993; Lyles and Gilbert, 1994; Darby and Creighton, 1995). However, the catalytic properties of these two sites are not identical. PDI with an intact N-terminal catalytic site has a K_m for reduced RNase of 29 μ M, which is four times the K_m of 7.1 μ M of PDI with an intact C-terminal catalytic site (Walker *et al.*, 1996). The concentration of reduced RNase used in our measurements of the isomerase activity was 8 μ M, which is very close to the K_m of the wild-type PDI. Therefore, mutations affecting binding to domain *a'* can be expected to have a marked effect on the isomerase activity as found here and in the previous study (Puig *et al.*, 1994) involving binding of the tripeptide probe to domain *a'* (see above). The concentration of insulin used in the reductase assay was much higher than that of RNase, being 160 μ M (Holmgren, 1979). Thus the two catalytic sites may operate equally in the disulfide reductase assay. The decreased activity values found with this assay with many mutants suggest that the mutations may also inhibit binding of insulin to domain *a'*.

The data indicating that the effects of various mutations on the different functions of the PDI polypeptide were not identical may also argue against any major overall structural changes. The $\Delta 455$ –457 deletion (SGG, the extreme C-terminal end of the predicted α helix) caused no inhibition of the subunit function and the disulfide bond reduction, for example, but had a definitive inhibitory effect on the chaperone function and the disulfide isomerase activity. Deletion $\Delta 431$ –433 (ASA) likewise had no inhibitory effect on prolyl 4-hydroxylase assembly or the chaperone function but had major effects on both the disulfide isomerase and reductase activities. Mutation F449R had major effects on the subunit function and the disulfide isomerase and reductase activities but no effect on the chaperone function, while F449W influenced only the subunit function. However, the 'local effects' of the

various mutations may well include destabilization of a region close to and around the mutation site.

It was reported very recently that deletion of the 51 most C-terminal amino acids from PDI totally prevented peptide binding and chaperone activities of the polypeptide (Dai and Wang, 1997). This deletion includes, in addition to domain *c*, the last α helix of domain *a'* and five residues of the presumed loop structure preceding the α helix, i.e. all the residues found here as critical for both the subunit function and chaperone activity. This deletion decreased the disulfide isomerase and reductase activities of the polypeptide by only 31 and 20%, respectively, indicating that it caused no major structural changes (Dai and Wang, 1997). This finding supports our data in that most of the mutations studied here may have caused mainly local effects.

The bacterial 21 kDa PDI-like protein DsbA recently has been reported to have chaperone activity similar to but weaker than that of PDI in increasing *in vitro* refolding and preventing aggregation of two proteins with no disulfide bonds (Zheng *et al.*, 1997). The crystal structure of DsbA shows that it resembles thioredoxin in spite of the low level of amino acid sequence identity (Martin *et al.*, 1993). A major difference is the presence of another domain that forms a cap over the -CGHC- catalytic site present in the thioredoxin-like domain of DsbA. This crystal structure indicates the presence of two grooves that may act as peptide-binding regions. The thioredoxin-like domain A has a long, relatively deep groove which is formed by lengthening of the loop connecting a β strand to the extreme C-terminal α helix of the domain (Martin *et al.*, 1993), with certain hydrophobic residues present in the loop preceding the α helix and in the α helix itself forming part of the lining of this groove. Interestingly, the predicted α helix identified here as critical for the various functions of PDI clearly corresponds to the α helix participating in groove formation in DsbA. Our attempts to model this region using the NMR structure of domain *a* of PDI (Kemink *et al.*, 1996) as the main reference suggest that the side chains of the most critical residues for the subunit function in this α helix are located on the hydrophobic side of the helix (details not shown), i.e. on the same side as the hydrophobic groove in DsbA. However, it seems very likely that many additional residues outside the region studied here must contribute to the peptide-binding region present in domain *a'*. In addition, the data of Klappa *et al.* (1998) and Darby *et al.* (1998) described above clearly indicate the need for domain *b'* for an effective binding. Previous studies with hybrid polypeptides containing regions of PDI and ERp60, a PDI-like polypeptide that also contains two thioredoxin-like domains in positions corresponding to those of the *a* and *a'* domains of PDI (Freedman *et al.*, 1994), likewise have demonstrated that although a region covering the last 72 amino acids of human PDI is essential for prolyl 4-hydroxylase tetramer assembly, this region is not sufficient (Koivunen *et al.*, 1996).

Materials and methods

PDI deletion constructs and generation of recombinant baculoviruses

Baculovirus transfer vectors for PDI Δ s were constructed by preparing two PCR fragments with pVL β as a template (Vuori *et al.*, 1992b) and

excluding the sequences to be deleted. The blunt-ended and kinase-treated PCR fragments coding for the N-terminus of PDI were digested with *EcoRI*, while those coding for the C-terminus were digested with *BamHI* and subsequently ligated to the *EcoRI*–*BamHI* site of pVL1392 (Invitrogen). The PDIΔ462–491 baculovirus transfer vector was constructed by synthesizing a PCR fragment with an artificial translation stop codon at position 462 and a *BamHI* restriction site at its 3' end using the His-PDI vector as a template (Annunen *et al.*, 1997). The fragment was digested with *EcoRI* and *BamHI* and ligated to the corresponding site of pVL1392.

The recombinant baculovirus transfer vectors were co-transfected into *Spodoptera frugiperda* Sf9 insect cells with a modified *Autographa californica* nuclear polyhedrosis virus DNA (BaculoGold, PharMingen) by calcium phosphate transfection (Gruenwald and Heitz, 1993). The resultant viral pools were collected 4 days later, amplified twice and used for recombinant protein production. Other recombinant baculoviruses used in this work were human α(I) and α(II), *C.elegans* α and human β coding for the corresponding prolyl 4-hydroxylase subunits (Vuori *et al.*, 1992b; Veijola *et al.*, 1994; Annunen *et al.*, 1997).

Site-directed mutagenesis

Individual amino acids in the region 437–454 of human PDI were subjected to mutation using an oligonucleotide-directed *in vitro* system (QuickChange™ Site-Directed Mutagenesis Kit, Stratagene). Plasmid S-138, containing the full-length cDNA for human PDI in pBluescript (Pihlajaniemi *et al.*, 1987), was used as a template. Following mutagenesis, the plasmids were digested with *NcoI* and *HindIII*, and the 766 bp fragment carrying the mutation site was cloned into the corresponding site of the baculovirus shuttle vector pFASTBAC1-PDI. The mutant sequences were verified by dideoxynucleotide sequencing. Recombinant baculoviruses were produced according to the BAC-TO-BAC system (Gibco-BRL). Viruses were harvested 72 h after infection, amplified twice and used for protein production.

E.coli expression vectors

PDI cDNA constructs coding for nine individual deletions and six individual point mutations along with a cDNA for the wild-type PDI were cloned into the pQE-31 *E.coli* expression vector below sequences coding for an N-terminal histidine tag (Qiagen). The cDNA for the wild-type PDI without its signal sequence was cloned into the *BamHI*–*HindIII* site of pQE-31 while all cDNAs for the deletion mutants, except that for PDIΔ462–491, were obtained by exchanging an *Apal*–*SmaI* fragment between the corresponding baculovirus transfer vectors and the wild-type cDNA in pQE-31. An *E.coli* expression vector for PDIΔ462–491 was generated by digesting the corresponding baculovirus transfer vector first with *BamHI*, then blunt-ending the site and digesting it again with *Apal*. The resulting fragment was then exchanged with the *Apal*–*SmaI* fragment of the wild-type cDNA in pQE-31. The point mutation constructs were obtained by exchanging an *Apal*–*HindIII* fragment between the corresponding baculovirus shuttle vector and the wild-type cDNA in pQE-31.

Analysis of recombinant proteins in insect cells

Sf9 cells were cultured in TNM-FH medium (Sigma) supplemented with 10% fetal bovine serum (Gibco-BRL) at 27°C, either as monolayers or in suspension. To produce recombinant proteins, insect cells seeded at a density of $0.7\text{--}1.5 \times 10^6/\text{ml}$ were infected with the recombinant viruses at a multiplicity of 5. The cells were harvested 72 h after infection, washed with a solution of 0.15 M NaCl and 0.02 M phosphate, pH 7.4, homogenized in a 0.1 M glycine, 0.1 M NaCl, 10 μM dithiothreitol (DTT) and 0.01 M Tris buffer, pH 7.8 (buffer A), supplemented with 0.1% Triton X-100, using one-twentieth of the original culture volume, and centrifuged at 10 000 g for 20 min. Aliquots of the resulting supernatants were analysed by 8% SDS–PAGE and non-denaturing 8% PAGE followed by Coomassie staining or Western blotting, and assayed for prolyl 4-hydroxylase activity. The remaining cell pellets were solubilized in 1% SDS in one-twentieth of the original culture volume and analysed by 8% SDS–PAGE. For protein purification, the cell homogenates in buffer A, containing 0.1% Triton X-100, were centrifuged at 160 000 g for 60 min, and the supernatants were applied to a Sephacryl S200 High Resolution gel filtration column (Pharmacia) equilibrated and eluted with buffer A. Absorbance of the fractions was measured at 280 nm, and several fractions were analysed by 8% SDS–PAGE. A sample pool was combined and applied to a Q-Sepharose column (Pharmacia), which was washed with buffer A until OD 280 nm was <0.02. The bound proteins were eluted with a gradient of buffer A and buffer A supplemented with 0.5 M NaCl. The fractions were analysed

as above, and those containing the purified recombinant proteins were pooled and ultrafiltered through a 10K membrane (Amicon). Concentrations of the insect cell-produced proteins were determined by OD at 230 nm and confirmed by densitometry of the corresponding protein bands analysed by SDS–PAGE.

Protein production and purification in E.coli

Production of recombinant proteins in *E.coli* was carried out in the SG13009[pREP4] strain at 37°C as suggested by the manufacturer (Qiagen). The cells were harvested after induction with isopropyl-β-D-thiogalactopyranoside (IPTG), homogenized in 50 mM Tris, pH 8, containing 0.1% Triton X-100 sonicated and centrifuged at 160 000 g for 1 h at 4°C. The supernatant was mixed with 5 ml of the Ni-NTA resin (Qiagen) and left on ice in a shaker at 4°C overnight. The resin was then transferred into a column and washed first with 3 vols of a buffer containing 5 mM imidazole and then with 10 vols of a buffer containing 60 mM imidazole. Elution was performed with a buffer of 0.5 M imidazole. Fractions of the eluate were analysed on an 8% SDS–PAGE under reducing conditions, and those containing the protein were pooled and applied to a PD-10 column (Pharmacia) equilibrated with 50 mM Tris, pH 8, to remove the imidazole. The purified protein was concentrated with Macrosep™ centrifugal concentrators (Filtron), and the protein concentration was measured with a Bio-Rad protein assay kit.

Enzyme activity assays

Prolyl 4-hydroxylase activity was assayed by a method based on the hydroxylation-coupled decarboxylation of 2-oxo[1-¹⁴C]glutarate (Kivirikko and Myllylä, 1982). Chaperone activity was measured by analysing the renaturation of denatured rhodanese in the presence of various concentrations of PDI or its mutant forms (Song and Wang, 1995). Regeneration of RNase activity from fully reduced RNase at a final concentration of 8 μM was measured as a continuous increase of the rate of hydrolysis of 2'3'-cCMP, a substrate for RNase as described by Lyles and Gilbert (1991). The rate of RNase reactivation was calculated using the extinction coefficients of 2'3'-cCMP and the product CMP, taking into account the effect of product inhibition. Concentration of the active RNase increases with a linear rate after a lag phase of a couple of minutes. The rate of increase in RNase activity in the presence of 1 μM mutant proteins was divided by the rate of reactivation exhibited by the wild-type PDI after background subtraction. Reduction of insulin was measured according to the DTT-dependent precipitation of insulin at 1 mg/ml followed by absorbance at 650 nm (Holmgren, 1979). The concentrations of the different PDI variants were 1 μM and the reaction proceeded at 37°C for 30 min.

CD spectrum analyses

CD spectrum measurements were carried out with a Jasco J500A spectropolarimeter in a 1 mm quartz cuvette. The molar ellipticity (θ) of PDI was calculated with a mean residue molecular mass of 110.

Secondary structure prediction and modelling

The secondary structure of human PDI was predicted by means of the PredictProtein server (EMBL, Heidelberg, Germany), which uses evolutionary information to predict secondary structure at >70% accuracy (Rost and Sander, 1993). The comparative modelling of the three-dimensional structure of the α' domain (residues D348–A461) was performed with the SWISSMODEL program (Peitsch 1996), using the OPTIMIZE function. The NMR structure of the α domain of human PDI (1MEK.pdb, Brookhaven Protein Data Bank; Kemmink *et al.*, 1996) was used as the main reference structure, while three structures of *E.coli* thioredoxin (1IXOA.pdb, XOB.pdb and 12TRX.pdb) were employed as other templates. All models were refined with CHARMM, and the figures were generated with the molecular graphics visualization tool RASMOL 2.5.

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