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Original Research Communication

Protein Disulfide Isomerases from *C. elegans* are Equally Efficient at Thiol–Disulfide Exchange in Simple Peptide-Based Systems But Show Differences in Reactivity Towards Protein Substrates

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ABSTRACT

Although the formation of disulfide bonds is an essential process in every living organism, only little is known about the mechanisms in multicellular eukaryotic systems. The reason for this uncertainty is that in addition to the well-known key enzyme protein disulfide isomerase (PDI), several PDI-like proteins are present in the ER of metazoans. In total, there are now 18 PDI-family members in the human endoplasmic reticulum, with different domain architectures and active site chemistries. To understand why multicellular organisms express multiple proteins with similarity to the archetypal mammalian PDI, the properties of three PDIs from the nematode *C.elegans* were investigated. Here the authors demonstrate that PDI-1, PDI-2, and PDI-3 show comparable kinetic properties in catalyzing thiol:disulfide exchange reactions in two simple peptide-based assays. However, the three enzymes exhibited clear differences in their reactivity towards protein substrates. The authors therefore propose that the three PDIs can catalyze similar thiol–disulfide exchange reactions in a substrate, but due to differences in substrate binding, they can direct a folding polypeptide chain onto different folding pathways and hence fulfil distinct and different functions in the organism. *Antioxid. Redox Signal.* 9, 1815–1823.

INTRODUCTION

The formation (oxidation) and rearrangement (isomerization) of disulfide bonds in proteins, resulting in the stabilization of the native structure, is a vital process in all organisms. *In vitro* studies have identified the enzyme protein disulfide isomerase (PDI) as one of the key players of this process in eukaryotes (4, 13). This enzyme catalyzes the formation of disulfide bonds in a folding polypeptide through its oxidant activity. Once disulfide bonds are formed, their correct spatial arrangement is ensured through the isomerase activity of PDI (11, 12, 14). PDI appears to be able to catalyze all of the steps in native disulfide bond formation for all substrate proteins reported. However, it is still unclear how PDI recognizes all of these different folding states, from unfolded through to substrates with conformations close to the native state, and yet does not appear to interact with native proteins.

PDI has the characteristic domain architecture **a-b-b'-a'-c** (6). The different domains of PDI have different functions. The **a** and **a'** domains contain the —WCGHC— active sites involved in catalyzing thiol–disulfide exchange reactions, the **b** domain is probably important for the overall structure of the molecule, and the **b'** domain is involved in substrate recognition and binding (9). While the **b'** domain contains the primary

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peptide or non-native protein binding site (19), the **a** and **a'** domains are also involved in substrate binding (19, 22). However, their binding affinities are apparently much lower and no direct peptide or non-native protein binding has been observed by **a** or **a'** in the absence of the **b'** domain. The negatively charged C-terminal part of PDI binds Ca^{2+} and contains the motif required for retention of the protein in the ER, but otherwise does not have any other function or activity (11).

In addition to its oxidoreductase activity, PDI is also the β-subunit of two hetero-oligomeric enzymes, namely prolyl 4-hydroxylase (21) and the microsomal triglyceride transferase complex (42). Whereas the latter is obligatory for the assembly of apoB-containing lipoproteins, the former is important in the post-translational formation of 4-hydroxyproline in collagen. In both complexes, PDI is required to prevent the aggregation of its partner subunits (20, 41). Site-directed mutagenesis studies have demonstrated that the active site cysteine residues of PDI are not essential for the assembly and activity of either complex (23, 39).

Multicellular systems contain several proteins with high similarity to archetypal mammalian PDI. In total, there are now 18 members of the PDI-family in the human ER, with a wide range of domain architectures and active site chemistries (for review, see Ref. 9). The presence of different catalysts of the same reaction in a multicellular system raises an important question: Why have these proteins evolved in the same intracellular compartment and what are their specific functions *in vivo*? One could speculate that all of these proteins catalyze different reactions. For example, some enzymes are very efficient at catalyzing the formation of a disulfide bond, while others are predominantly involved in the isomerization of already existing disulfide bonds. However, several PDI-like proteins (*e.g.*, ERp72, ERp57, P5, and archetypal PDI), show similar *in vitro* enzyme activities [*i.e.*, depending on the redox environment they can catalyze disulfide bond formation, reduction, and/or isomerization (1, 7)] and therefore it is likely that these enzymes catalyze similar reactions *in vivo*.

Another explanation for the existence of similar enzymes within the same compartment is that they might have different substrate specificities and hence catalyze the same reaction (*i.e.*, formation of native disulfide bonds) in different substrates. For at least one PDI-family member, this has been demonstrated, with the substrate binding site in the **b'** domain of Erp57 having become specialized for the interaction with the ER-resident lectins calreticulin and calnexin (16, 29, 32), giving it a specificity for glycoproteins (25).

To address the question of why there are several members of the PDI family expressed in metazoans, we studied the properties of PDI-like proteins from the nematode *Caenorhabditis elegans*. This organism expresses three proteins, PDI-1, PDI-2, and PDI-3, which have the same domain architecture and which show significant sequence similarity to archetypal mammalian PDI (PDI-1 and PDI-2) and ERp57 (PDI-3; (10)). There is some evidence that these three PDI family members have different functions *in vivo*. First, there are multiple lines of evidence pointing towards the involvement of PDI-2 in prolyl hydroxylation (26). Second, while the temporal and cell-specific expression of PDI-1 is similar to that of PDI-2 (28), a genomic knockout of *pdi-1* does not show any observable phenotype (http://aceserver.biotech.ubc.ca/cgi-bin/stable/strain.pl?cl%20ass=Strain;name=VC586, accessed May 26, 2007), indicating that this protein is not essential under the tested conditions. Third, PDI-3 has been reported to be involved in the maintenance of the body morphology, probably by stabilization of the extracellular matrix through disulfide bond formation, chaperone activity, or transglutaminase-like cross-linking activity (10).

To distinguish whether the three PDIs from *C. elegans* catalyze different enzymatic reactions in the same substrate or whether they catalyze similar reactions, but act on different substrates, we characterized their properties using small peptides and proteins as substrates.

MATERIALS AND METHODS

"Scrambled" ribonuclease A, bovine insulin, the homobifunctional cross-linking reagent disuccinimidyl glutarate (DSG), and all other chemicals were obtained from Sigma (Poole, UK). [^{125}I] Bolton–Hunter labeling reagent, ECL-reagent, and X-ray films were purchased from Amersham GE Healthcare (Little Chalfont, UK). Mastoparan (INLKALAALAKKIL), Δ-somatostatin (AGSKNFFWKTFTSS), peptide P (PTIKFFWGDTASPK), glutathionylated substrate peptide SQLWC(glutathione)LSN, and the peptide used in the oxidation assay (NRCSQGSCWN) were synthesized at the Biomolecular Science Facility, Department of Biosciences, University of Kent, Canterbury, Kent, UK, as described previously for other peptides (18). [^{125}I] Bolton–Hunter labeling of peptides was performed as recommended by the manufacturer.

Maintenance of C. elegans *strains*

The commonly used N2 Bristol strain was grown on Nematode Growth Medium, seeded with *E. coli* strain OP50, at 18°C, as described previously (36).

Cloning of pdi-1, pdi-2, and pdi-3 genes from cDNA

RNA isolation from *C. elegans* mixed stage population were carried out as described previously (2). cDNA was generated with the Retroscript kit from Ambion (Huntingdon, UK) as instructed by the manufacturer. The following sets of primers were used (Qiagen, Hilden, Germany):

PDI-1F: GGGGGGgctagcGACAGCGAAAACGTGCTT;
PDI-1R: GGGGGGcggccgTTAAAGCTCTTCGTGATCCTGAG;
PDI-2F: GGGGGGgctagcGCCGTCATTGAAGAAGAAGAG;
PDI-2R: GGGGGGcggccgTTAGAGCTCGGTGTGTCCCTC;
PDI-3F: GGGGGgctagcGGAGGAGCCGTTCTCGAGTATACC;
PDI-3R: GGGGGcggccgTTACAATTCAGTCTTCTTCTTCTTCTTTC.

The primers allowed the insertion of a *NheI* site at the amino-terminus and a *EagI* site at the carboxy-terminus (*lowercase*)

The primers complementary to the 3'-end included a stop codon. The PCR products were cloned into a pET28a vector (Novagen, Beeston, UK). The constructs were designed such that mature proteins, without the signal sequences, were generated. For ease of purification and detection, a hexa-histidine tag was engineered onto the amino-terminus of the mature proteins. All constructs were verified by DNA sequencing.

Protein production

Protein production was carried out in *E. coli* strain BL21 (DE3) carrying the pLysS plasmid to control leak-through expression and to allow subsequent cell lysis by freeze/thawing. The cells were incubated at 30°C in LB medium containing 25 μg/ml kanamycin and 25 μg/ml chloramphenicol. Four hours after induction with 1 m*M* isopropyl-thio-β-D-galactopyranoside the cells were harvested by centrifugation.

The cell pellets were suspended in buffer A (20 m*M* Na-phosphate pH 7.3) and DNAse I (Sigma) to a final concentration of 10 μg/ml was added. After freeze/thawing the suspension twice, the lysate was cleared by centrifugation at 12,000 *g* for 30 min. Filtered supernatants were loaded onto a Ni-NTA column (3 ml volume, GE Healthcare, Little Chalfont, UK), equilibrated with buffer A. Unspecifically bound proteins were removed by washing the column with buffer A containing 500 m*M* NaCl and 50 m*M* imidazole, followed by buffer A. Recombinant *C. elegans* PDIs were eluted with buffer A containing 10 m*M* EDTA. The elution fractions were loaded onto a ResourceQ column (6 ml, Amersham Biosciences, UK) equilibrated with buffer A and eluted with a linear gradient from 0 to 0.5 *M* NaCl in buffer A. Fractions containing *C. elegans* PDIs were checked for purity by SDS-polyacrylamide gel electrophoresis, and fractions containing pure protein were pooled and the buffer was replaced with 20 m*M* sodium phosphate buffer, pH 7.3 using ultrafiltration [Biomax-15k nominal molecular mass membrane filter (Millipore, Watford, UK)]. The correct molecular mass of all purified proteins was verified by electrospray–ionization (ESI) mass spectrometry (Micromass, Manchester, UK). Human PDI was purified as described previously (24).

Peptide-based enzymatic assays

Peptide oxidation assay. The method of Ruddock *et al*. (33) using a fluorescent decapeptide NRCSQGSCWN was used to determine the catalytic ability of each of the purified *C. elegans* PDIs to form a disulfide bond in a peptide. Buffer A supplemented with 150 m*M* NaCl was placed in a fluorescence cuvette to give a final assay volume of 1 ml. To this was added 10 μl of oxidized glutathione (50 m*M* stock solution in buffer A), 20 μl of reduced glutathione (100 m*M* stock solution in buffer A), and 5–10 μl of enzyme, final concentration of 100 n*M*. After mixing, the cuvette was placed in a spectrophotometer (Perkin-Elmer Life Sciences, Shelton, UK, model LS50) for 5 min to allow thermal equilibration of the solution. 6.3 μl of substrate peptide (539 μ*M* stock solution in 30% acetonitrile, 0.1% trifluoroacetic acid) was added, the sample was quickly mixed, and the change in fluorescence intensity (excitation at 280 nm, emission at 350 nm, slit widths at 5 nm) was monitored over an appropriate time (usually 15 min) with 600–1,800 data points being collected. The formation of an intramolecular disulfide bond in the peptide is accompanied by an 18% decrease in its fluorescence intensity. In the absence of substrate, no significant change in fluorescence occurred. Initial rates of the reaction were determined from linear fits over 20 arbitrary units of change in fluorescence signal or other applicable linear part of the curve and correlated to the total change in fluorescence observed during the reaction. The first few data points were ignored due to extra noise being observed during the first few seconds. All experiments were carried out in triplicate.

Assay for deglutathionylation activity. The method of Peltoniemi *et al*. (29) using the fluorescent glutathionylated substrate peptide SQLWC(glutathione)LSN was used to determine the deglutathionylation activity of each of the purified *C. elegans* PDIs. Fluorescence measurements were performed with a spectrometer (Perkin-Elmer Life Sciences model LS50B) using a 315 μl cuvette. Assays were carried out in buffer A, including 5 μ*M* glutathionylated peptide substrate, 1 m*M* EDTA, 1 m*M* GSH, 50 μ*M* NADPH, 20 n*M* glutathione reductase, and 200 n*M* PDIs. To prevent loss of glutathione reductase due to adsorption on the walls of plastic tubes and the cuvette, dilutions of glutathione reductase were made into solutions containing bovine serum albumin (final concentration 1 μg/ml). All measurements were done at 25°C, excitation 280 nm, emission 356 nm, and slit widths 5 nm (29). All experiments were carried out in triplicate.

Protein-based assays

Insulin precipitation assay. To determine the reductase activity of the three PDIs from *C. elegans*, the precipitation of the insoluble B-chain of bovine insulin was measured spectrophotometrically (35, 37). In a total volume of 200 μl 10 m*M* DTT and 1 μ*M* of the respective PDI were incubated for 5 min in Buffer A supplemented with 150 m*M* NaCl. The reaction was started by addition of 20 μl of 10 mg/ml bovine insulin and the precipitation of the B-chain was monitored at 540 nm in a Helios spectrophotometer (Perkin-Elmer Life Sciences) over 16 min.

BPTI production and refolding assay

Mature BPTI with an additional methionine residue as the first amino acid was constructed from synthetic oligonucleotides and subcloned as an NdeI/BamHI fragment into pET23 (Novagen).

BPTI production was carried out in *E. coli* strain BL21(DE3) pLyS grown in LB medium at 37°C and induced at an A_{600} of 0.3 for 3 h with 1 m*M* isopropyl β-D-thiogalactoside. The cells were pelleted at 8,000 rpm for 10 minu. The supernatant was removed and the pellet was resuspended in 1/10 the volume of buffer A. and DNase I (Sigma) was added to a final concentration of 10 μg/ml. Lysis of bacteria was carried out by freeze/thawing the samples twice. The insoluble cell debris was collected by centrifugation (8,000 rpm for 20 min). The cell pellet was washed twice with 50 m*M* Tris, 10 m*M* EDTA, 0.5% Triton-X100, pH 8, and twice with distilled water. The cell pellet was solubilized in 50 m*M* Tris-buffer, pH 8.75, containing 5 *M*

guanidinium chloride and 20 m*M* DTT, and incubated at room temperature for 45 min. The sample was filtered through a 0.45 μM filter before being applied to a SOURCE 5 RPC ST 4.6/150 column (Amersham Biosciences, Uppsala, Sweden) from which the protein was eluted with a linear gradient from buffer 1 (2% ACN, 0.1% TFA) to 60% of buffer 2 (90% ACN, 0.1% TFA) over 20 column volumes. Pure protein containing fractions were lyophilized and then suspended into 10 m*M* HCl, pH 2.

The correct mass of BPTI was verified by ESI-mass spectrometry. Reduced BPTI (50 μM) was alkylated with iodoacetamide (1.1 *M* in buffer A) and purified with PepClean C-18 spin column (Pierce, Rockford, IL), before mass spectrometry to verify that BPTI was in the reduced form.

For the refolding assay 70 μM reduced BPTI, 10 μM of the various PDIs, 2 m*M* reduced glutathione, and 0.5 m*M* oxidized glutathione were incubated in refolding buffer (0.1 *M* Na-phosphate, 1 m*M* EDTA, pH 6.0). The reaction was stopped by addition of iodoacetamide (1.1 *M* in buffer A) and BPTI and its folding intermediates were purified with a PepClean C-18 spin column (Pierce) before ESI-mass spectrometry analysis. It should be noted that different species may bias their detection by ESI-mass spectrometry and therefore the results are only semiquantitative.

Chemical cross-linking of peptides and "scrambled" RNAse

The binding of radiolabeled peptides and scRNAse by the PDIs was carried out as described previously (18, 19). Cross-linking was performed with the homobifunctional cross-linking reagent disuccinimidyl glutarate (DSG). The samples were supplied with 1/5 volume of cross-linking solution (2.5 m*M* DSG in buffer A, supplemented with 150 m*M* NaCl). The reaction was carried out for 60 min at 0°C. Cross-linking was stopped by the addition of SDS-PAGE sample buffer. The samples were subjected to electrophoresis in 12.5% SDS polyacrylamide gels with subsequent autoradiography for radiolabeled peptides or by Western blot analysis using an anti-hexa histidine antibody (Amersham Biosciences, UK) for experiments using scRNAse. The detection was carried out with ECL-reagent (Amersham Biosciences, UK).

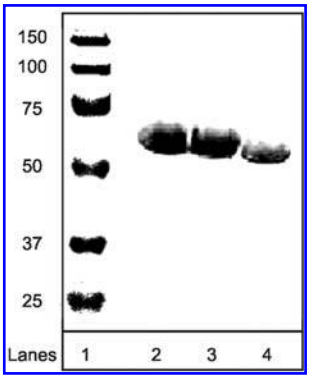

FIG. 1. Purification of *C. elegans* PDIs. PDI-1, -2, and -3 were purified as described in Material and Methods. After elution from ResourceQ column, protein-containing fractions were pooled and buffer exchanged. To look for small amounts of contaminating proteins 15 μg of purified proteins were loaded onto a 12% reducing SDS-polyacrylamide gel. Staining was carried out with Coomassie Blue. No additional bands were seen, indicating the purity of the protein preparations. M, molecular weight markers.

TABLE 1. COMPARISON OF ENZYMATIC ACTIVITIES BETWEEN PDIS

	Half time of oxidation (sec)	*Half time of deglutathionylation (sec)*
PDI-1	53 ± 5	116 ± 7
PDI-2	50 ± 4	110 ± 6
PDI-3	56 ± 5	100 ± 7
Human PDI	55 ± 6	ND
uncatalysed	750 ± 15	1400 ± 20

RESULTS

To investigate whether the three *C. elegans* PDIs have similar enzymatic properties, we first cloned, recombinantly expressed, and purified them to apparent homogeneity (Fig. 1). Subsequently we tested their activities in peptide-based assays as well as in assays using model substrate proteins.

Protein disulfide isomerases PDI-1, PDI-2, and PDI-3 catalyze similar enzymatic reactions in simple peptide-based assays

To investigate whether the three different PDIs from *C. elegans* show similar enzymatic properties, their oxidant activities were measured in a peptide-based assay, described by Ruddock *et al*. (33). The peptide-based activity assays provided the opportunity to study the enzymatic properties of the three PDIs independent of their individual substrate specificities, since it has been shown that peptides shorter than 12 amino acids do not significantly interact with the primary substrate binding site in the **b'** domain of PDI (25). A decrease in the fluorescence, indicative of disulfide bond formation between the two cysteine residues in the test peptide, was measured in the presence and absence of PDIs, and the half times of the reactions were calculated (Table 1). The results clearly demonstrate that PDI-1, -2, and -3 catalyzed the formation of the disulfide bond in the test peptide compared with the noncatalyzed control and that they did so with comparable kinetics to that of purified human PDI.

A similar result was observed when the deglutathionylation activities of the three PDIs were compared, using the test peptide SQLWC(glutathione)LSN (27; Table 1). Deglutathionylation of peptides and proteins has previously been shown for human PDI family members (27). In essence, it is an isomerization reaction, with a peptide (or protein) in a mixed disulfide with glutathione reacting with reduced glutathione to give a reduced thiol group on the peptide and oxidized glutathione. Since noncatalyzed oxidation of peptides and proteins by oxidized glutathione proceeds via a peptide–glutathione mixed disulfide, it is an important thiol–disulfide exchange reaction during pro-

tein folding as well as during oxidative stress (27). All three PDIs were able to catalyze deglutathionylation of the peptide and showed comparable activities, catalyzing deglutathionylation with similar kinetics.

From these experiments, we conclude that there are no significant differences in the catalytic activities of PDI-1, -2, and -3 when small peptides are used as substrates (*i.e.*, that the thiol—disulfide exchange activities of their **a** and **a'** domains were comparable with these two peptide substrates).

Protein disulfide isomerases PDI-1, PDI-2, and PDI-3 show different reactivities towards protein substrates

The peptide-based assays for thiol–disulfide exchange used peptides which do not interact significantly with domains of PDI other than the catalytic **a** or **a'** domains. We therefore addressed the question whether the three different PDIs from *C. elegans* showed similar enzymatic properties in assays which require the **b'** domain of PDI. This domain contains the primary non-native protein binding site and this is known to be specialized between PDI-family members (9, 18, 32).

The insulin-precipitation assay is commonly used to analyze the reductase activity of oxidoreductases (35, 37) (*i.e.*, their ability to reduce disulfide bonds to dithiols). Upon reduction of the disulfide bonds of insulin with dithiothreitol, the insulin B chain aggregates and precipitates while the A chain remains soluble. In the presence of oxidoreductases, the initial lag-phase of the aggregation is significantly shortened compared to the uncatalyzed reaction. All three *C. elegans* PDIs reduced the lag-phase of the precipitation of the insoluble B-chain (Fig. 2), indicating that all are active in the reduction of insulin. However, the lag-phase for PDI-2 was significantly shorter, implying that PDI-2 is more active in this assay than either PDI-1 or PDI-3 (i.e., that towards this protein substrate there were clear differences in the catalytic properties of these three PDIs).

To confirm and extend our observation that the three PDIs showed different properties when assayed with protein substrates, we employed reduced, unfolded bovine pancreatic trypsin inhbitor (BPTI) as a substrate. The refolding pathway of BPTI is one of the best characterized model systems for non-catalyzed disulfide bond formation (5, 40). It has been reported that in order to reach the native conformation with three intramolecular disulfides, BPTI has to undergo isomerization reactions between protein species containing two non-native disulfide bonds (5). Hence the use of BPTI as a substrate allows the investigation of the oxidation and isomerization activities of PDI family members, with mammalian PDI being able to catalyze all steps in the reaction. In this assay, the kinetics of the catalyzed refolding of denatured and reduced BPTI was measured in the absence and presence of the different PDIs. At set timepoints, the reactions were quenched by the addition of a large excess of iodoacetamide. The reaction of iodoacetamide with free thiol groups adds 57Da to the mass of the protein and hence the various disulfide-bonded forms of BPTI, at each timepoint, could be resolved by mass spectrometry. Similary the presence of glutathionylated BPTI could be detected by the additional 305Da mass.

The analysis showed that all three PDIs were able to catalyze disulfide bond formation in BPTI. Initially one disulfide bond was formed with this species disappearing as the second disulfide bond was formed (Fig. 3A). For both PDI-1 and PDI-3, the one disulfide species was clearly detectable only at the 2.5 min timepoint, while the uncatalyzed reaction resulted in maximal accumulation of this species after 10 min. In contrast, the PDI-2 catalyzed reaction proceeded so quickly to the two disulfide bond-containing species that no one disulfide bond containing species was detectable at any timepoint measured (Fig. 3A). With all three PDIs, we detected intermediates with two disulfide bonds, which were converted into the native three disulfide form at a faster rate than the noncatalyzed rate (Figs. 3B and C).

Three significant differences were observed between the reactions catalysed by the *C. elegans* PDIs. First, the conversion of the two disulfide form to the three disulfide form was significantly faster for the PDI-2 catalyzed reaction than for that catalyzed by PDI-1 or PDI-3 (Fig. 3C). Second, we observed a significant accumulation of a glutathionylated two-disulfide bonded species in the presence of PDI-3 (Fig. 3D). Third, only PDI-2 was able to catalyze to near completion the formation of the native three disulfide bonded form of BPTI. Even after 24 h, PDI-1 and PDI-3 were only able to generate 67% and 71%, respectively, of the three disulfide bonded species. This was, however, still significantly higher than the 30% of three disulfide bonded species for the uncatalyzed reaction.

Taken together these results indicate that the three PDIs from *C. elegans* catalyze the refolding of reduced BPTI. However,

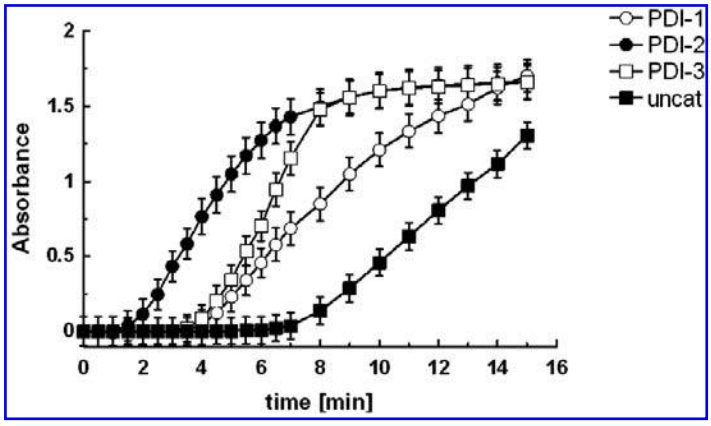

FIG. 2. Precipitation of insulin in the presence of PDIs. In a total volume of 200 μl, 10 m*M* DTT and 1 μ*M* of the respective PDI were incubated for 5 min in Buffer A supplemented with 150 m*M* NaCl. The reaction was started by addition of 20 μl of 10 mg/ml bovine insulin, and the precipitation of the B-chain was monitored at 540 nm in a Helios spectrophotometer. A sample without PDIs served as a control. Experiments were carried out in triplicate. Means and standard errors are shown.

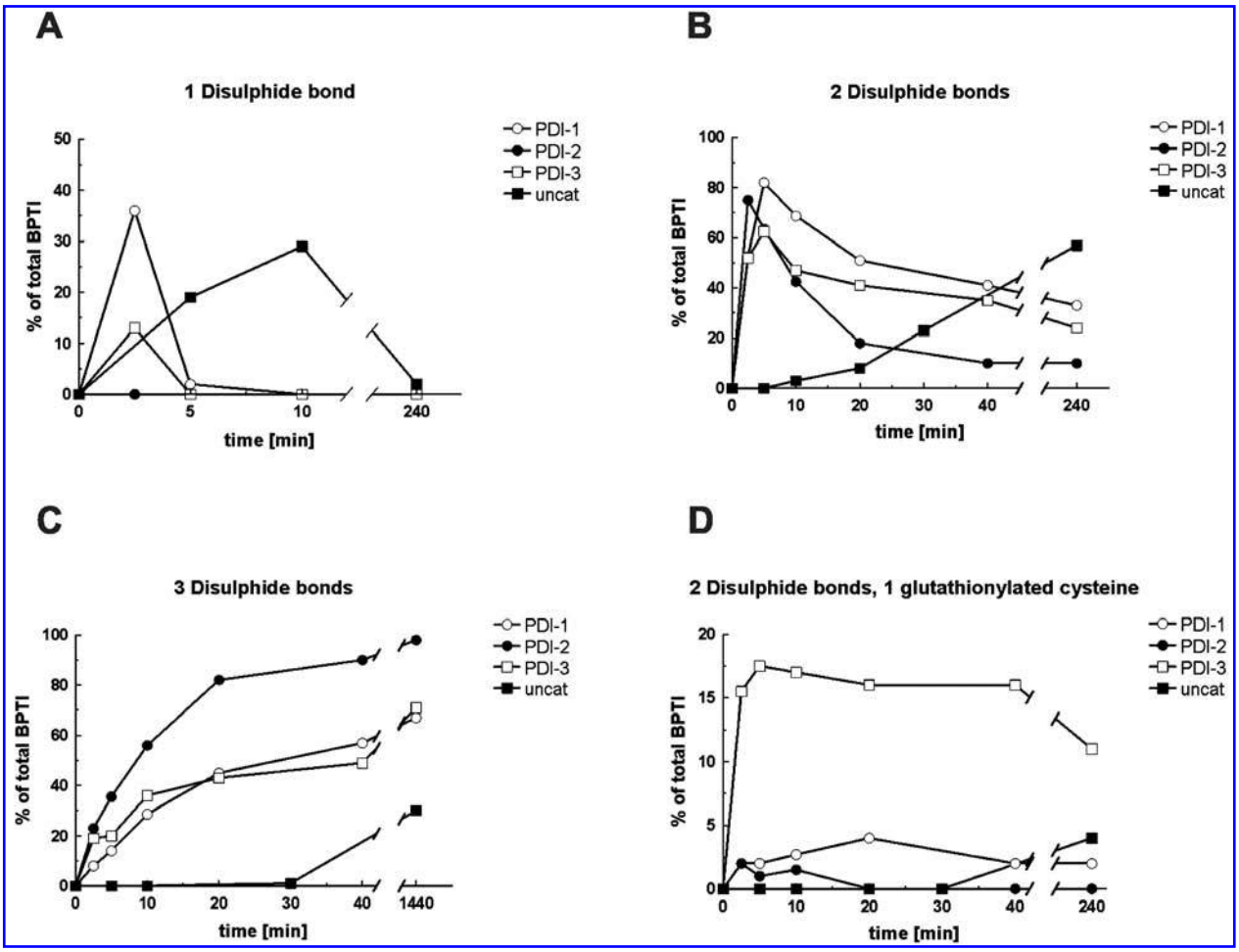

FIG. 3, Refolding of reduced BPTI in the presence of PDIs. 70 μM of reduced BPTI was added to refolding buffer (0.1 *M* Na-phosphate, 1 m*M* EDTA, 2 m*M* reduced glutathione, 0.5 m*M* oxidized glutathione, pH 6.0) in the presence or absence of 10 μM PDIs. At set timepoints, aliquots were withdrawn and the reaction quenched by the addition of 1.1 *M* iodoacetamide. The samples were purified with PepCelan C-18 spin-column and then the products analyzed by ESI-mass spectrometric analysis. The reaction of each free thiol group with iodoacetamide added 57 Da to the mass of the protein, while glutathionylation added 305 Da. This mass adducts allowed the determination of BPTI species that contained no disulfides, one disulfide, two disulfides, or the native three disulfides, plus the determination of glutationylated species. Experiments were carried out in duplicate and the means are shown. During the reaction, reduced BPTI is first converted into one disulfide-containing species, then this is subsequently oxidized to two disulfide-containing species which are in turn converted to the native three disulfide-containing species. Note that some of the different possible two disulfide-containing species are able to proceed to the native state even in the absence of a catalyst of isomerization, while others absolutely require a catalyst such as PDI. (**A**) Kinetic profile of the appearance and subsequent disappearance of BPTI containing one disulfide bond and four reduced cysteine residues. (**B**) Kinetic profile of BPTI containing two disulfide bonds and two reduced cysteine residues. (**C**) Kinetic profile of BPTI containing three disulfide bonds. (**D**) Kinetic profile of BPTI containing two disulfide bonds, one reduced cysteine residue, and one glutathionylated cysteine residue.

from the differences in the rate of formation of the various disulfide bonded forms and the accumulation of a different species in the PDI-3 catalyzed reaction, we hypothesize that the refolding of BPTI is catalyzed by the various PDIs via different folding pathways.

Interaction of PDI proteins with model peptides and 'scrambled' ribonuclease

Since the various PDIs from *C. elegans* led to the accumulation of different amounts of intermediates in the refolding of reduced BPTI, we speculated that this is due to different ways by which the various PDIs interacted with the substrate protein.

To confirm this hypothesis, we employed a well-established cross-linking approach to examine substrate binding by the **b'** domains of PDI-family members and radiolabeled peptides (18). As shown in Fig. 4A, PDI-1, -2, and -3 all interacted with the three model peptides tested, although clear differences in the interaction patterns were noticeable. While PDI-2 showed similar cross-linking intensities with all three peptides, PDI-1 exhibited stronger interaction with Δ-somatostatin than with Mastoparan and peptide P. The interactions observed between

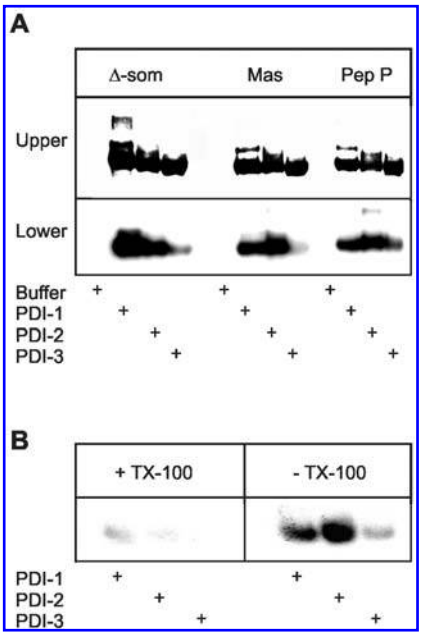

FIG. 4. Cross-linking of [^{125}I] Bolton–Hunter labeled peptides to purified PDIs. [^{125}I] Bolton–Hunter labeled Δ-somatostatin (Δ-som), Mastoparan (Mas), or peptide P (Pep P) (33 μM) were incubated with 3.3 μM PDI-1, PDI-2, PDI-3 in buffer A, supplemented with 150 mM NaCl for 10 min at 0°C in a total volume of 10 μl. A sample without protein served as a control. Samples were subsequently incubated with DSG (final concentration 0.5 mM) for 60 min at 0°C. After cross-linking, the samples were analyzed on 10% polyacrylamide gels. (**A**) Representative Coomassie Blue staining (*upper panel*) and autoradiography (*lower panel*) of cross-linking of peptides to PDIs. (**B**) Representative autoradiography of peptide P binding in the presence and absence of 0.1% Triton X-100.

PDI-3 and peptides were weaker than with PDI-1 or PDI-2. The interactions between PDIs and model peptides were specific since heat-inactivation of the PDIs prior to incubation with the peptides abolished the interactions (data not shown). The intensities of the cross-linking products between the different PDIs and peptides were strongly reduced in the presence of Triton X-100, indicating that hydrophobic interactions are important for the efficient binding of peptides to the PDIs tested (Fig. 4B). These results showed that all three PDIs from *C. elegans* interacted specifically with model peptides and that hydrophobic interactions were an important factor in the binding process, but that the three PDI family members showed differences in specificity of substrate binding using peptides which interact with the **b'** domain of PDI (18).

To investigate whether this specificity in binding manifested itself also at the level of binding to substrate proteins, we carried out chemical cross-linking of the PDIs and a misfolded protein, specifically 'scrambled Ribonuclease' (scRNAse). As shown in Fig. 5, we found that while all three *C. elegans* PDIs gave a specific cross-linking product, the signal was much weaker with PDI-3.

Taken together, our results clearly indicate that the various PDIs from *C. elegans* exhibit different interaction profiles towards model substrates. We therefore conclude that the differences in the reactivities observed in the protein-based enzymatic assays are reflected in the interactions between the different PDIs and their substrates.

DISCUSSION

The formation of native disulfide bonds in many secreted and membrane-bound proteins is essential for their correct three-dimensional structure and hence their biological activities. Native disulfide bond formation occurs in the endoplasmic reticulum of eukaryotes, an organelle that provides the appropriate environment for this process (3, 16). In addition to an oxidizing environment, the ER also contains several enzymes that have been shown to facilitate disulfide bond formation in folding polypeptide chains. For many years, archetypal mammalian PDI has been recognized as the key enzyme involved in the generation of native disulfide bonds, however, numerous enzymes with significant sequence similarity to PDI have been described (9). The presence of these catalysts within the same intracellular compartment raises the important question about their biological function: Do they catalyze different reactions in the same substrate or do they catalyze the same enzymatic reaction in different substrates?

To address this question, we analyzed the *in vitro* properties of three different proteins with high sequence similarity to archetypal mammalian PDI from the same organism, namely the nematode *C. elegans*. The three PDI-like proteins PDI-1, PDI-2, and PDI-3 all have a similar domain architecture **a-b-b'-a'-c** with the **a** and **a'** domains containing the catalytically active sites comprising the thioredoxin-motif —WC*XX*C—. All three proteins have an N-terminal signal sequence and a C-terminal ER-retention motif. The most noticeable differences in the amino acid sequences of these proteins are in the **b** and **b'** domains. This observation is important in the understanding of the biological functions of these proteins, since it has been shown that the **b'** domain of human PDI contains the primary substrate binding domain (19) and that this site in human ERp57

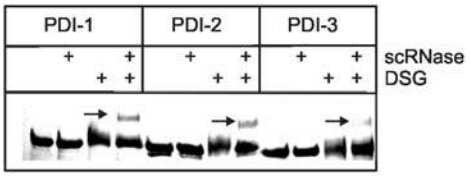

FIG. 5. Interaction of PDIs with 'scrambled' RNase. Purified PDI-1, PDI-2, and PDI-3 (3.3 μM in buffer A, supplemented with 150 mM NaCl) were incubated with 100 μM 'scrambled' RNase' (scRNase) or buffer A prior to cross-linking with DSG. A sample without PDIs served as a control. After cross-linking, the samples were analyzed on 10% polyacrylamide gels with subsequent electro-transfer onto PVDF membranes. The samples were probed with an anti-hexa-histidine primary antibody, raised in mouse, and a secondary anti-mouse antibody, conjugated to horseradish peroxidase and visualized by ECL. Cross-linking products are indicated by an *arrow*, uncross-linked proteins are indicated by an *asterisk*. A representative blot is shown.

has become specialized for the interaction with the ER-resident lectins calreticulin and calnexin (17, 31, 34).

Database analysis of the expression profiles of the three *C. elegans* PDIs indicated that all three proteins are expressed throughout the entire life cycle of the nematode (15), (*i.e*., there is no stage-specific expression of one of the proteins detectable. Furthermore, serial analysis of gene expression profiles indicate that none of the *C. elegans* PDIs shows cell-specific expression (http://www.wormbase.org/db/seq/sage?name=SAGE%3Agtgcggacattgcaagc;class=SAGE_tag, accessed May 26, 2007). From these data we conclude that all three proteins are expressed in the ER of the nematode at the same time.

To address the question whether the **a** and **a'** domains of three *C.elegans* PDIs could catalyse the same reaction, we used small peptides as substrates. In these reactions, peptide oxidation or deglutathionylation is predominantly facilitated by an interaction between the active sites of the catalyst and the substrate with little or no involvement of other domains. Hence these measurements are independent of the substrate specificities of the **b'** domains. The three PDIs from *C. elegans* catalyzed the oxidation of cysteine residues in the model peptide substrate with similar kinetics (Table I). Likewise, similar kinetic profiles were observed when the deglutathionylation of another model substrate peptide was measured. From these results, it can be concluded that the catalytic domains of the three PDIs from *C. elegans* can catalyze the same thiol-exchange reactions with comparable kinetics and efficacies.

However, when we used model substrates that were able to potentially interact with the **b'** domain, we found significant differences in the enzymatic properties of the three PDIs. PDI-2 showed the highest activity in the insulin precipitation assay. A similar observation was made when the refolding of denatured and reduced BPTI was investigated (Fig. 3): PDI-2 was the most effective catalyst of disulfide bond formation, while PDI-1 and PDI-3 were less efficient and did not oxidize BPTI to completion even after 24 h. Furthermore, in the presence of PDI-3, we detected significant accumulation of a monoglutathionylated product. The most likely explanation for this difference is the way PDIs interact with their substrates such that they promote different folding pathways.

To address whether the difference between the three PDIs is due to the substrate specificities of the **b'** domain primary substrate binding site, we investigated their interaction with radiolabeled model peptides which do not interact with other domains of PDI (18). In our cross-linking-based experiments, we saw no particular substrate specificity for PDI-2 (Fig. 4A) (*i.e*., all three model peptides were bound with similar efficacy). However, we observed differences in the efficacy of interaction with the different model peptides for PDI-1 and PDI-3, with PDI-3 showing weak interaction signals for all three peptides tested (Fig. 4A). This result indicates that the different PDIs have different specificities for model peptides. To demonstrate that this is not only true for small model peptides, we also analyzed the interaction between the three PDIs and 'scrambled' Ribonuclease, a misfolded substrate protein. Again our results showed differences between the three PDIs with PDI-1 and PDI-2, but not PDI-3 interacting strongly with the substrate (Fig. 5). Substrate specificity has also been observed for other members of the PDI family. For example, ERp57 cannot replace PDI in the prolyl 4-hydroxylase complex, highlighting the specific function PDI plays in the formation of this complex (30). ERp57 has been shown to act *in vitro* and *in vivo* specifically on glycosylated substrates through its exclusive interaction with the ER-resident lectins calnexin and calreticulin (8, 27). This interaction arises as the **b**' domain of ERp57 is specifically adapted for lectin association rather than substrate binding (32). In this context, it is interesting to note that PDI-3 is more homologous to ERp57 than to PDI and hence the weak interaction with peptide and protein substrates may be a reflection of the specialization of its **b'** domain to interact with *C. elegans* lectins. However, it should be noted that human ERp57 precipitates when added to reduced BPTI in the absence of its interaction partners (38), while PDI-3 does not. Another example for substrate specificity was described for the pancreas-specific enzyme PDIp. In this case it was shown that the interaction of PDIp with substrates is restricted to tyrosine and tryptophan residues in the substrate (32).

Taken together, our results show that the three different PDIs from *C.elegans* can catalyze the same thiol exchange reactions with similar kinetics, but differ in their substrate specificity and interact with different intermediates during the folding of a polypeptide chain, thus promoting different folding pathways.

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ABBREVIATIONS

ACN, acetonitrile; BPTI, bovine pancreatic trypsin inhibitor; *C. elegans*, *Caenorhabditis elegans*; DSG, disuccinimidyl glutarate; DTT, dithiotreitol; EDTA, ethylenediaminetetraacetic acid; ER, endoplasmic reticulum; GSH, glutathione; PDI, protein disulfide isomerase; RNAi, RNA-mediated gene silencing; scRNAse, "scrambled" Ribonuclease A; TFA, trifluoracetic acid.

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