A 21-kDa C-terminal Fragment of Protein-disulfide Isomerase has Isomerase, Chaperone, and Anti-chaperone Activities*

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A catalyst of disulfide formation and isomerization during protein folding, protein-disulfide isomerase (PDI) has two catalytic sites housed in two domains homologous to thioredoxin, one near the N terminus and the other near the C terminus. The thioredoxin domains, by themselves, can catalyze disulfide formation, but they are unable to catalyze disulfide isomerizations (Darby, N. J. and Creighton, T. E. (1995) Biochemistry 34, 11725-11735). A 21-kDa, C-terminal fragment of PDI (amino acids 308-491), termed weePDI, comprises the C-terminal third of the molecule. The $k_{\text{cat}}$ for ribonuclease oxidative folding by weePDI is 0.26 ± 0.02 min$^{-1}$, 3-fold lower than the wild-type enzyme but indistinguishable from the activity of a full-length mutant of PDI in which both active site cysteines of the N-terminal thioredoxin domain have been mutated to serine. Eliminating the ability of weePDI to escape easily from covalent complexes with substrate by mutating the active site cysteine nearer the C terminus to serine has a large effect on the isomerase activity of weePDI compared with its effect on the full-length enzyme. weePDI also displays chaperone and anti-chaperone activity characteristic of the full-length molecule. As isolated, weePDI is a disulfide-linked dimer in which the single cysteine (Cys-326) outside active site cross-links two weePDI monomers. The presence of the intermolecular disulfide decreases the activity by more than 2-fold. The results imply that the functions of the core thioredoxin domains of PDI and other members of the thioredoxin superfamily might be modified quite easily by the addition of relatively small accessory domains.

Protein-disulfide isomerase (PDI) is an abundant, 55-kDa resident of the endoplasmic reticulum (ER) (1-3). The enzyme catalyzes a variety of thiol/disulfide exchange reactions, including the formation and rearrangement of disulfide bonds during oxidative protein folding. PDI possesses two domains that are homologous to the redox active protein, thioredoxin (4). One is located near the N terminus, and another is found near the C terminus. Each of the domains is characterized by a redox-active dithiol/disulfide site with the general sequence CGHC. Thioredoxin domains, including active-site CXC sequences, have been found in a number of redox-active proteins, suggesting that these proteins constitute a thioredoxin superfamily with versatile thiol/disulfide exchange activities including oxidation, reduction, and isomerization.

Individual members of the thioredoxin superfamily have up to three thioredoxin domains linked together. Erp73 has three thioredoxin domains (6), whereas Erp61 has two (7). Other members of the thioredoxin superfamily such as thioredoxin itself, glutaredoxin (8), or the Escherichia coli periplasmic protein, DsbA (9), have only one thioredoxin homology domain. All of these single domain proteins are poor catalysts of disulfide rearrangements, suggesting that domains other than the thioredoxin domains contribute significantly to substrate interactions that are important for disulfide rearrangements.

PDI displays an amazingly large range of interactions including association with folded proteins, with unfolded proteins, and with small molecules. It serves as an essential subunit of prolyl hydroxylase (10) and triglyceride transferase (11), two multi-subunit proteins of the ER. The catalytic activity of PDI is not essential to its function as a structural subunit of prolyl hydroxylase (12). PDI can also interact with unfolded proteins in a number of ways. At concentrations in large excess over its unfolded protein substrate, PDI can function as a chaperone and inhibit aggregation (13) even for substrate proteins that do not form disulfide bonds (14, 15). With unfolded proteins that also self-aggregate, substoichiometric concentrations of PDI can, surprisingly, facilitate aggregation (anti-chaperone activity), presumably by interacting with and non-covalently cross-linking smaller aggregates (13, 15, 16). Because of the multiple interactions that PDI can accommodate, the non-thioredoxin domains of the protein may be involved in interactions with other proteins.

The cysteine residues of the two CXCC centers of the thioredoxin domains are responsible for the disulfide isomerase activity of PDI; a mutant PDI with all four active-site cysteines changed to serines has $\leq 0.5\%$ of the activity of the wild-type enzyme in catalyzing ribonuclease oxidative folding (17). At RNase concentrations near and below the $K_m$, the two active sites contribute almost equally to catalysis of RNase refolding (10). However, when surveyed over a much larger range of substrate concentrations, the two PDI active sites contribute unequally to catalysis. Neither active site is totally essential for disulfide isomerase activity; however, the N-terminal active site contributes more to catalysis at saturating substrate, whereas the C-terminal active site contributes more to the...
binding of substrate in the steady-state (17). At each thioredoxin domain active site, the cysteine that is closer to the N terminus interacts with the substrate to form a covalent intermediate (18). PDI mutants with only a single active site cysteine (at the more N-terminal position) do not catalyze substrate oxidation, but still have significant activity in catalyzing disulfide rearrangements. The more C-terminal cysteine at each active site provides a mechanism to reduce substrate disulfides and enables PDI to escape from slowly rearranging complexes (18).

When expressed independently, the N-terminal thioredoxin domain (residues 1–120) folds into a stable species, and the structure has been solved by NMR (19). The C-terminal thioredoxin domain (residues 348–462) can also be expressed, but it is somewhat less stable than the N-terminal domain. Each of the isolated thioredoxin domains is catalytically active in the oxidation of protein substrates, but neither isolated thioredoxin domain exhibits significant activity in catalyzing isomerization (20), often the rate-limiting step in the oxidative refolding of disulfide containing proteins. Recent characterization of a PDI fragment consisting of the first 257 residues, which encompasses the N-terminal thioredoxin domain and a substantial portion of the central region of the molecule, showed that this substantial fragment was also inactive in catalysis of disulfide isomerization (21). Obviously, sequences in the C-terminal region of the protein other than the thioredoxin domain are essential for catalysis of rearrangements.

During the expression of recombinant PDI in *E. coli*, we and others (22, 23) noted the co-expression of a 21-kDa fragment of PDI. N-terminal sequencing indicates that the fragment results from alternative translation initiation at the first internal AUG, located at Met-307. Here, we report that this C-terminal fragment of recombinant rat PDI (residues 308–491, termed *wee*PDI) has substantial activity in catalyzing disulfide isomerization during the oxidative refolding of reduced RNase. The *k*<sub>cat</sub> is comparable to that of a full-length PDI in which the N-terminal domain has been inactivated by mutation of the active site cysteines to serine. The activity of this C-terminal fragment suggests that the essential features that impart isomerization activity to PDI, even the isomerization activity of the N-terminal thioredoxin domain, reside in short segments flanking the C-terminal thioredoxin domain.

**EXPERIMENTAL PROCEDURES**

**Materials**—Glutathione (GSH), glutathione disulfide (GSSG), 3′,5′-cCMP, dithiothreitol, bovine pancreatic ribonuclease A (RNase), equine liver alcohol dehydrogenase, TPCK-treated trypsin, HEPES, and cells from Sigma. DEAE-Sephadex and chelating Sepharose (fast flow) liver alcohol dehydrogenase, TPCK-treated trypsin, HEPES, and cells from Sigma. DEAE-Sephacel and chelating Sepharose (fast flow) were from Pharmacia Biotech Inc. All other reagents were analytical grade.

**Methods**—UV-visible spectrophotometry was performed on either a Beckman DU70 or DU7500 spectrophotometer with the cell compartment maintained at 25 °C. SDS-PAGE (12.5% acrylamide) was performed by the method of Laemmli (24), and proteins were detected using the method maintained at 25.0 °C. SDS-PAGE was performed on a Beckman 110A gradient system using a Kratos variable wavelength UV-visible detector.

**Expression and Purification**—The expression of *wee*PDI accompanies the expression of full-length PDI when expression is induced from expression plasmid (pET-PDI.2) (26). *E. coli* strain BL21(DE3) (Novagen, Madison, WI) was transformed with a plasmid encoding the wild-type PDI or various cysteine mutants (18). Overnight cultures from a single colony were grown on LB to an *A*<sub>600</sub> of 0.8–1.0 and induced with 0.4 mM isopropyl-1-thio-β-D-galactopyranoside (final concentration). After overnight growth, cells were harvested by centrifugation, disrupted as described previously (26), dialyzed, and applied to a DEAE-Sephacel column (2.5 × 20 cm). *wee*PDI was separated from wild-type PDI using a 2-liter linear gradient (total volume) of 0–0.5 M NaCl in 0.025 M sodium phosphate, pH 6.3. *wee*PDI and the various *wee*PDI mutants eluted after the corresponding full-length PDI. Fractions containing predominantly *wee*PDI were verified by SDS-PAGE and stained.

The wild-type *wee*PDI (SS) was purified further by ion-exchange (DEAE-5PW, Bio-Rad) HPLC with a gradient of 0.15–0.5 M NaCl in 0.025 M sodium phosphate buffer, pH 6.3, over 30 min. SDS-PAGE showed that the highly purified *wee*PDI was greater than 95% homogeneous and contained <1% wt/PDI. For the mutant *wee*PDIs, the final traces of full-length PDI were removed by HPLC gel filtration (7.5 × 600 mm, TSK-9300SW, CEL Associates, Inc, Houston, TX) after reduction with 10–20 mM DTT at pH 7.5–8.0 for at least 2 h. The solvent for gel filtration was 25 mM phosphate buffer (pH 6.3) containing 0.2 M NaCl.

**Kinetics of RNase Refolding**—Protein-disulfide isomerase activity was determined using a previously published continuous assay for catalysis of the refolding of reduced, denatured RNase (27). Briefly, reduced, denatured RNase was added to a cuvette containing a thiolone rebox buffer (1 mM GSH, 0.2 mM GSSG), the RNase substrate, cCMP (4.5 mM), and the indicated concentrations of *wee*PDI and RNase in 0.1 M Tris-acetate buffer (pH 8.0) containing 2 mM EDTA. The temperature was maintained at 25.0 °C. After adding reduced RNase to initiate the reaction, the absorbance at 296 nm was continuously monitored by cCMP hydrolysis was monitored continuously (0.5-min intervals) for 30 min. The amount of refolded RNase at any time was calculated from the first derivative of the absorbance with respect to time after correction for substrate depletion and product inhibition, as described (27). Parallel controls with no PDI were performed at each RNase concentration and subtracted from the rate of the catalyzed reaction. Control rates were generally <25% of the rate of the catalyzed reaction. At RNase concentrations greater than 30 μM, an additional control was performed in which cCMP was omitted from the assay to correct for the catalysis of RNase aggregation (17). The correction for aggregation was generally small compared with the catalyzed reaction (<5% at concentrations of RNase of 40 μM). Activities are reported in units of micromolar active RNase formed per min. Data were combined and analyzed by non-linear least squares fitting (28) to a rectangular hyperbola. The reported error limits are the parameter standard deviations calculated from the fitting algorithm.

**Chaperone and Anti-chaperone Activity**—The final yield of enzymatically active lysozyme was determined after oxidative refolding of reduced denatured lysozyme at pH 7.0 (HEPES), 37 °C as described previously (3). The refolding buffer containing 3 mM GSH and 0.5 mM GSSG in 0.1 M HEPES, 20 mM NaCl, 2 mM EDTA, and 5 mM MgCl<sub>2</sub>. The final lysozyme concentration was 10 μM. Yields, measured when the production of native lysozyme had reached a constant value, are based on the activity exhibited by the same concentration of native lysozyme. Alcohol dehydrogenase aggregation was induced by thermally denaturing 10 μM alcohol dehydrogenase at 40 °C. Aggregation was followed in a Beckman DU 7500 spectrophotometer by measuring the absorbance at 360 nm due to light scattering caused by increased turbidity. Alcohol dehydrogenase was added to temperature-equilibrated buffer (0.05 M HEPES, pH 7.0) containing PDI to initiate aggregation. An apparent steady-state in absorbance with time as described previously (15).

**Peptide Mapping/Disulfide Formation**—*wee*PDI (1.0 mg/ml) was digested by incubating with 2% (v/w) TPCK-treated trypsin at pH 8.0 (0.1 M Tris-HCl, 2 mM EDTA) containing 5 mM CaCl<sub>2</sub> for 4–6 h at 37 °C. After a further addition of 2% trypsin (v/w), the incubation was continued overnight. The digested sample was mixed in 1% trifluoroacetic acid and loaded onto an appropriately charged and washed Sep-Pak C<sub>18</sub> cartridge, desalted with 0.1% aqueous trifluoroacetic acid, and eluted with acetonitrile/water/trifluoroacetic acid (80:20:0.1). After drying under nitrogen, the digested, desalted sample was resuspended in water/trifluoroacetic acid/formic acid (99:0.25:1), and an aliquot (10 μl) was analyzed by packed capillary HPLC-electrospray mass spectrometry. The capillary HPLC consisted of two Waters prototype high pressure syringe pumps controlled by a Waters model 680 gradient control.
Results

Expression and Purification—Inducing the expression of recombinant rat PDI (55 kDa) also indicates the expression of a smaller protein (21 kDa) (Fig. 1). The 21-kDa band cross-reacts with anti-PDI antibodies (data not shown) and has an N-terminal sequence of TKYKPESDeltaEKI. This sequence begins immediately after the first internal ATG codon of the PDI gene, as described by Kemmink and co-workers (19). The two thioether domains containing the active sites are labeled α and β. The internally homologous linker domains are labeled b and ε, and the acidic C-terminal domain is labeled c after Edman and colleagues (4).

Expression of RNase—Like wild-type PDI, weePDI catalyzes the oxidative refolding of reduced, denatured RNase, catalyzing both oxidation and isomerization of RNase disulfides. At saturating reduced RNase, the $k_{cat}$ based on a 21-kDa monomer, is 0.26 ± 0.02 min⁻¹ (Fig. 3). A mutant weePDI in which the active site cysteine nearer the C terminus had been

mutated to serine (weePDI(SO)) showed detectable activity in the isomerization of RNase (Fig. 3). However, the activity was considerably lower than that of the wild-type weePDI. Because of the low activity, the $K_m$ for RNase could not be determined accurately; however, the $K_m$ was only 10% that of weePDI and only 1% that of the wild-type, full-length enzyme. A mutant weePDI molecule with no active site cysteines showed no detectable activity over background, even at high concentrations (32 μM). Kinetic constants are summarized in Table I.

During the RNase oxidative folding assay, weePDI gains activity (Fig. 4). With increasing time during the assay, the activity of weePDI increases by at least 2.3-fold (Fig. 4, inset). Examination of the weePDI on non-reducing gels (Fig. 1, inset) shows that the weePDI as isolated is a disulfide-linked dimer of 42 kDa. The glutathione reductase buffer (1 mM GSH and 0.2 mM GSSG) is sufficient to reduce the intermolecular disulfide of weePDI at catalytic weePDI concentrations (<3 μM). SDS-PAGE of N-ethylmaleimide-quenched refolding reactions indicates that the reduction is essentially complete within 10 min (data not shown), suggesting that the activity increase is associated with reduction of the intermolecular disulfide linking the two weePDI molecules. When RNase refolding is initiated
with weePDI (2.8 μM) that has been prerduced by DTT, RNase refolding is initially faster than when the reaction is initiated with the disulfide-linked dimeric enzyme.

**Peptide Mapping/Disulfide Formation—Full-length**, wild-type PDI has six cysteine residues (4), whereas weePDI has three (Fig. 2). With an odd number of cysteine residues, the possibility exists for the formation of intermolecular disulfides, and this proved to be the case. Reaction of the protein with 5,5’-dithiobis(2-carboxynitrobenzoiate) (32) in 6 M guanidine HCl indicates that there are <0.1 free sulphydryl groups per weePDI (21 kDa) as isolated. HPLC-electrospray mass spectral analysis of tryptic peptides of weePDI (Table II) shows the presence of a fragment with a molecular mass of 2915.4 Da, consistent with the presence of a symmetrical disulfide involving Cys-326. A peptide corresponding to the mass of the active-site containing fragment was also observed with a molecular mass of 1796.8 Da, two mass units smaller than predicted for a dithiol-containing peptide of the expected sequence, suggesting that the active site cysteines are disulfide-bonded to each other. In addition, no ionic series were detected for the other potential disulfide-linked peptides. Thus, weePDI monomers are linked by a Cys-326–Cys-326 intermolecular disulfide.

Gel filtration HPLC indicates that weePDI has an apparent molecular mass of 126 ± 16 kDa under non-reducing conditions and that reduction with DTT before HPLC shifts the apparent molecular mass to 52 ± 2 kDa (data not shown). However, apparent molecular masses by gel filtration are dependent on the hydrodynamic behavior and shape of the protein as well as potential interactions with the column matrix. To determine a true molecular mass, sedimentation equilibrium was performed on weePDI under reducing and non-reducing conditions. Under non-reducing conditions, the molecular mass determined by sedimentation equilibrium is 41.5 ± 0.2 kDa, consistent with a simple dimeric structure (Fig. 5). In the presence of 10 mM DTT, sedimentation equilibrium analysis yielded an apparent molecular mass of 20.7 ± 0.2 kDa, consistent with the behavior of weePDI as a covalent dimer that dissociates into a monomeric species upon reduction of the intermolecular disulfide. Thus, the apparent molecular masses observed by HPLC gel filtration do not reflect the true association state of the molecule, suggesting that both the weePDI monomer and dimer are elongated molecules.

**Chaperone and Anti-chaperone Activity**—In addition to its disulfide isomerase activity, full-length PDI exhibits chaperone activity. At high concentrations, PDI inhibits the aggregation of proteins that form disulfides (13, 16) and also proteins that do not form disulfides (14, 15). A rather surprising property of PDI is its ability to facilitate the formation of aggregates of some unfolded proteins (13, 15, 16). This “anti-chaperone” activity occurs with unfolded proteins that have an intermediate tendency to self-aggregate and only at low concentrations of PDI relative to the unfolded protein. Anti-chaperone activity is observed under both oxidizing (13, 16) and reducing conditions (15). The stimulation of aggregation at low concentration and the inhibition of aggregation at high concentration has been compared with the classical immunoprecipitation of a multivalent protein with a bivalent antibody (15). weePDI, like its full-length counterpart, exhibits chaperone activity at high concentration and anti-chaperone activity at low concentration during the refolding of hen lysozyme (Fig. 6). The increase in lysozyme yield observed at high weePDI concentrations is significantly lower than that observed with wtPDI, consistent with the observation that isomerase activity is required for chaperone activity during lysozyme refolding (13). Like its full-length counterpart, weePDI also shows chaperone and anti-chaperone activity with thermally denatured alcohol dehydrogenase (Fig. 6), increasing the aggregation at low concentrations and decreasing aggregation at high concentrations.

**DISCUSSION**

As a folding assistant in vitro, the C-terminal third of PDI is capable of performing all of the known foldase and chaperone functions of the full-length PDI molecule. The $k_{cat}$ of 0.26 min$^{-1}$ for weePDI is about one-third that of the wild-type enzyme; however, it is identical to the $k_{cat}$ of a full-length PDI mutant in which the catalytic activity of the N-terminal thioredoxin domain is inactivated by mutation of the active-site cysteines to serine (PDI(ROSS)), Table I) rather than by deletion of the

![Fig. 3. Initial velocity of weePDI-catalyzed RNase oxidative folding as a function of RNase concentration. The assay was performed at pH 8.0, 0.1 M Tris-acetate, 2 mM EDTA in a glutathione redox buffer composed of 1 mM GSH and 0.2 mM GSSG. Data are shown for weePDI(SS) (●) and for weePDI(SO) (○). The concentration of weePDI (SS) was 1.6 μM and weePDI(SO) was 6.6 μM. The solid curve for weePDI(SS) is a rectangular hyperbola drawn with a $K_m$ of 0.26 min$^{-1}$ and a $K_v$ of 24 μM. The solid curve for weePDI(SO) is a rectangular hyperbola drawn with a $K_m$ of 0.026 min$^{-1}$ and a $K_v$ of 24 μM.](image)

**TABLE I**

**Kinetic properties of weePDI and wild-type PDI in the oxidative folding of RNase**

All experiments were performed at 25 °C at pH 8.0 in 0.1 M Tris-acetate buffer containing 2 mM EDTA. Error estimates represent the standard deviation of the parameter estimates from non-linear least squares fitting to a hyperbolic function.

| PDI          | $k_{cat}$ (μM) | $K_m$ (RNase) | $k_{cat}/K_m$ | Percent of weePDI(SSSS) |
|--------------|----------------|---------------|---------------|------------------------|
| weePDI(SS)   | 0.26 ± 0.02    | 24 ± 4        | 1.1           | 10                     |
| weePDI(SO)   | (0.026)*       | (24)*         | 0.1           | 1                      |
| weePDI(OO)   | 0.76 ± 0.02    | 6.9 ± 0.8     | 11            | 100                    |
| weePDI(SSSS) | 0.24           | 7.1           | 3.4           | 31                     |
| weePDI(OSSO) | 0.082 a        | 8.7           | 0.95          | 9                      |
| weePDI(OOSO) | <0.03**        |               |               | 0.25                   |

* Estimates assuming that the $K_m$ for weePDI(SO) is identical to that for weePDI(SS).

**The data for wtPDI and mutants of wtPDI are taken from Ref. 18.**
Fig. 4. The effect of reduction on the weePDI-catalyzed oxidative folding of reduced, denatured RNase. weePDI (--) or weePDI prescissed for 1.5 h with 1 mM DTT (--) was used to catalyze the oxidative folding of RNase. The background reaction (---) was conducted in the absence of weePDI. The RNase concentration was 8.0 μM, and the weePDI was at 2.8 μM. Refolding was conducted at pH 8.0 (0.1 M Tris-acetate, 2 mM EDTA), 25 °C in a glutathione redox buffer (1 mM GSH, 0.2 mM GSSG). The inset shows the ratio of the concentration of RNase formed at a given time as catalyzed by weePDI that was initially oxidized to that catalyzed by weePDI that was initially reduced. Data shown are the average of two experiments. Non-linear least squares curve fitting of the data in the inset to a single exponential function (solid curve) yields an initial ratio of 0.38 ± 0.01, a final ratio at infinite time of 0.88 ± 0.005, and a first-order rate constant of 0.13 ± 0.01 min⁻¹.

Table II

| Peptide                  | Expected mass | Found |
|--------------------------|---------------|-------|
| Cys-326-Cys-326          | 2915.4        | +     |
| ITQFCHFLEGK              |               |       |
| Cys-326-Cys-380/Cys-383 | 3255.5        | -     |
| ITQFCHFLEGK              |               |       |
| NVFGFYAPWGCHCK           | 3595.6        | -     |
| Cys-380/Cys-3-Cys-380/Cys-383 |          |       |
| NVFGFYAPWGCHCK           | 1796.8        | +     |
| NVFGFYAPWGCHCK           |               |       |

Fig. 5. Sedimentation equilibrium behavior of weePDI and wild-type PDI. Sedimentation equilibrium was performed in 0.05 M HEPES, 0.2 mM NaCl, pH 7.0. A, weePDI in the absence of reducing agents (○). B, weePDI in the presence of 10 mM DTT (●).

TABLE II

| Peptide                  | Expected mass | Found |
|--------------------------|---------------|-------|
| Cys-326-Cys-326          | 2915.4        | +     |
| ITQFCHFLEGK              |               |       |
| Cys-326-Cys-380/Cys-383 | 3255.5        | -     |
| ITQFCHFLEGK              |               |       |
| NVFGFYAPWGCHCK           | 3595.6        | -     |
| Cys-380/Cys-3-Cys-380/Cys-383 |          |       |
| NVFGFYAPWGCHCK           | 1796.8        | +     |
| NVFGFYAPWGCHCK           |               |       |

entire N-terminal domains (17). The $K_m$ for RNase is only 3.4-fold higher for weePDI than for full-length PDI. Thus, there are few essential interactions in either the a or b domains (or most of the b' domain) with substrate undergoing catalysis of rearrangement at the C-terminal thioredoxin domain. However, the converse is not true; elements in the C-terminal part of the molecule are clearly required for the isomerization activity of the N-terminal thioredoxin domain. Expressed by itself, the isolated N-terminal thioredoxin domain is an active oxidase, but has no detectable isomerase activity (20). However, a full-length mutant PDI with an inactive C-terminal thioredoxin domain, wtPDI(SSSO), has near wild-type oxidase and isomerase activities at saturating RNase (17). Darby and co-workers (21) extended the sequence of the N-terminal domain to include the N-terminal portion of the first linker do-

main (residues 1–257) without generating any detectable isomerase activity. Darby and Creighton (20) have also expressed and isolated the C-terminal thioredoxin domain (a'), but, like the N-terminal domain, it is an active oxidase but inactive isomerase. Clearly, the properties of weePDI establish that substrate isomerization by either of the two thioredoxin domains requires essential elements located outside the thioredoxin domains in the C-terminal third of the molecule.

PDI interacts with its substrates using one or more nonspecific peptide/protein binding site that have broad substrate specificity (33). A peptide photoaffinity label identified only a single binding site near the C terminus (34). Since this region is also present in weePDI, it is possible that the increased $K_m$ for weePDI reflects a structural perturbation of the single C-terminal peptide binding site induced by the deletion of the N terminus and linker regions. Alternatively, there may be multiple binding interactions between PDI and large protein substrates, including interaction sites in the N-terminal region or linker region.

Recently, it has been possible to separate the disulfide formation activity and the disulfide isomerase activity of PDI. Mutants of PDI with a single active site cysteine will catalyze substrate isomerization, but not substrate oxidation (18, 35). With the full-length enzyme, a single cysteine located at the C-terminal active site, wtPDI(OOSO), catalyzes RNase isomerization with a $k_{cat}/K_m$ that is about 30% that of a full-length PDI with a mutated N-terminal thioredoxin domain, wtPDI(OSS). Like its full-length counterpart, the single-cysteine version of weePDI(SSO) also catalyzes disulfide isomerization. However, weePDI(SO) has only 10% of the activity of the corresponding wild-type active site, weePDI(SS), suggesting that binding contributions from the N-terminal domains become more important for the catalytically impaired enzyme.

As isolated, weePDI is a covalent dimer in which two 21-kDa monomers are linked by an intermolecular disulfide involving Cys-326. Reduction increases the disulfide isomerase activity
of the two monomers in a head-to-head fashion decreases the ability of the catalytic centers to function efficiently. With wild-type PDI, the two thioredoxin domains are linked head-to-tail (Fig. 2), an arrangement that does not lead to a significant interference between the activities of the two thioredoxin domains. Thus, the connecting region and the head-tail linkage between the two thioredoxin domains in wild-type PDI may minimize the interference between the two thioredoxin domains.

Truncation of PDI does not obliterate the chaperone and anti-chaperone functions of the molecule, which are mediated by non-covalent interactions between PDI and the unfolded protein substrate (13–16). For lysozyme refolding, the chaperone activity leading to increased formation of native lysozyme requires the isomerase activity (13); however, to simply maintain substrate solubility, the catalytic activity of the enzyme is not required (15, 36). A comparison of the activity of wtPDI and weePDI is consistent with a C-terminal location for the major interactions with the substrate, even for the N-terminal active site. Consequently, it is not surprising that the chaperone and anti-chaperone activities also reside in the C-terminal portion of the molecule.

The thioredoxin domains of PDI are, by themselves, poor catalysts of disulfide isomerization (20). However, including a limited amount of the C-terminal region of the molecule in weePDI restores near wild-type isomerization activity. There appears to be little advantage to simply stringing thioredoxin domains together; the head-to-head arrangement of two closely juxtaposed C-terminal thioredoxin domains actually diminishes the activity of the dimeric weePDI. The relatively low disulfide isomerase activity of Erp72, a thioredoxin family member that resides in the ER and has three thioredoxin domains (6), confirms that more is not necessarily better with respect to thioredoxin domains. A recent NMR structure of the b domain of PDI (37) indicates that the b domain has an overall fold that is very similar to the thioredoxin fold, suggesting that PDI is composed of catalytically active thioredoxin domains separated by two catalytically inactive thioredoxin domains. It is interesting that the disulfide-forming catalyst of the bacterial periplasm, DsbA, has a small insert that disrupts the middle of the thioredoxin domain and adds a small, hydrophobic patch above the dithiol/disulfide active site (38).

In summary, a small, C-terminal fragment of PDI comprising only a third of the molecule is an effective catalyst of disulfide formation and rearrangement and exhibits both chaperone and anti-chaperone activity like its full-length counterparts. Although the thioredoxin domains of PDI and related proteins are essential to their functions as catalysts, the addition of appropriate accessory domains modifies this versatile redox catalytic domain to provide different functions during oxidative protein folding.

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