Zinc-dependent dimerization of the folding catalyst, protein disulfide isomerase

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Abstract

Protein disulfide isomerase (PDI), an essential folding catalyst and chaperone of the endoplasmic reticulum (ER), has four structural domains (**a-b-b'-a'**) of approximately equal size. Each domain has sequence or structural homology with thioredoxin. Sedimentation equilibrium and velocity experiments show that PDI is an elongated monomer (axial ratio 5.7), suggesting that the four thioredoxin domains are extended. In the presence of physiological levels (<1 mM) of Zn^{2+} and other thiophilic divalent cations such as Cd^{2+} and Hg^{2+} , PDI forms a stable dimer that aggregates into much larger oligomeric forms with time. The dimer is also elongated (axial ratio 7.1). Oligomerization involves the interaction of Zn^{2+} with the cysteines of PDI. PDI has active sites in the N-terminal (**a**) and C-terminal (**a'**) thioredoxin domains, each with two cysteines (CGHC). Two other cysteines are found in one of the internal domains (**b'**). Cysteine to serine mutations show that Zn^{2+} -dependent dimerization occurs predominantly by bridging an active site cysteine from either one of the active sites with one of the cysteines in the internal domain (**b'**). The dimer incorporates two atoms of Zn^{2+} and exhibits 50% of the isomerase activity of PDI. At longer times and higher PDI concentrations, the dimer forms oligomers and aggregates of high molecular weight (>600 kDa). Because of a very high concentration of PDI in the ER, its interaction with divalent ions could play a role in regulating the effective concentration of these metal ions, protecting against metal toxicity, or affecting the activity of other (ER) proteins that use Zn^{2+} as a cofactor.

Keywords: zinc; protein disulfide isomerase; disulfide; oligomerization; protein folding

Protein disulfide isomerase (PDI) is a ubiquitous, essential enzyme found in the endoplasmic reticulum (ER) of all eukaryotes. PDI catalyzes a set of disulfide-exchange reaction that includes oxidation of sulfhydryl groups into disulfides, reduction of disulfides, and rearrangement of disulfide bonds to correct folding mistakes. PDI also has chaperone activity and is found as a subunit of several multi-subunit enzymes. The catalytic site of PDI consists of a dithiol/disulfide center that undergoes redox state changes during catalysis (Gilbert 1997).

PDI has four structural domains (**a-b-b'-a'**), each with sequence and/or structural homology to the small redox protein, thioredoxin. The two catalytic domains (**a** and **a'**) each

have active site sequences CGHC, and two additional cysteines are located in the internal, **b'** domain (Edman et al. 1985). The "internal" cysteines do not contribute to PDI activity; replacement of all four active site cysteines with serines completely eliminates the disulfide-exchange activity of PDI (Lyles and Gilbert 1994), and replacing the **b'** cysteines with serines does not affect catalytic activity.

PDI, even when highly purified, may form a set of heterogeneous molecular species under various conditions (Pace and Dixon 1979). PDI purified from bovine liver microsomes contains dimers, and possibly higher molecular weight species, that are linked by disulfide bonds (Carmichael et al. 1979; Pace and Dixon 1979). It has also been noted that PDI that is homogeneous on SDS-PAGE can be separated into two species on gel-filtration or ion-exchange HPLC (Hu and Tsou 1992). The smaller species was found to be more catalytically active. At first, the heterogeneity was attributed to C-terminal proteolysis, but later, the same investigators found that the two components of PDI prepa-

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rations had the same amino acid composition. Gel filtration indicated that they were tetramers and dimers with apparent molecular masses of 240 kDa and 120 kDa, respectively (Yu et al. 1994).

In our laboratory, PDI is normally purified by a two-step technique that involves chromatography on Zn^{2+} -chelating Sepharose (Gilbert et al. 1991). It was noticed that under certain conditions, Zn^{2+} and other thiophilic divalent cations (Cd^{2+} , Hg^{2+}) cause the association of PDI into higher-molecular-weight species. In addition, Baksh et al. (1995) reported that PDI interacts with calreticulin, another ER-resident protein, in a Zn -dependent manner. The Zn^{2+} -dependent association with calreticulin and the long-standing observations of various oligomeric forms of PDI, prompted an investigation of the interactions of Zn^{2+} with PDI. In this article, we find that Zn^{2+} at physiological concentrations (~ 1 mM; Alfaro and Heaton 1974) inhibits the isomerase activity of PDI by half and induces the formation of PDI dimers and higher-order aggregates by non-covalently cross-linking an active site cysteine with a cysteine in the **b'** domain. We have also found that PDI and its dimer are very elongated and show anomalous molecular weights by gel filtration.

Results

Treatment of reduced rat PDI (sequence molecular mass of 55 kDa) with mM concentrations of Zn^{2+} at pH 7.0 (50 mM

Figure 1. Oligomerization of PDI in the presence of Zn^{2+} . PDI ($4.4 \mu\text{M}$) was incubated with 1 mM zinc acetate at room temperature for the indicated time and then separated by gel-filtration HPLC. Initially (0 h, *top left*), PDI was all monomeric. After 1 h, three peaks could be observed (*top right*), corresponding to aggregate (A), dimer (D), and monomer (M). After 3 or 24 h of incubation (*bottom*), more PDI is converted into dimer and eventually aggregates.

Figure 2. Dependence of the disappearance of PDI monomer on the concentration of Zn^{2+} . PDI ($20 \mu\text{M}$) was incubated in 50 mM HEPES (pH 7.0) with different concentrations of Zn^{2+} for 17 h at room temperature, and the amount of residual monomer determined by gel-filtration HPLC is shown. The curve is a rectangular hyperbola with a $\text{Zn}_{1/2}$ of 0.2 ± 0.03 mM.

HEPES) results in the formation of higher-molecular-weight species (Fig. 1). Although the sequence molecular mass is 55 kDa, gel-filtration HPLC calibrated against protein molecular mass standards shows that PDI that has not been treated with Zn^{2+} displays an apparent molecular mass of 108 ± 9 kDa. After incubation with Zn^{2+} , species with molecular masses of 236 ± 30 kDa and >600 kDa appear. With increasing Zn^{2+} concentration, an increasing amount of the 108 kDa disappears and is converted to higher-molecular-weight species (Fig. 2). The time course of the oligomerization (Fig. 3) shows that with time, the low-molecular-weight species are converted increasingly to large aggregates (>600 kDa). Other thiophilic divalent cations (Hg^{2+} and Cd^{2+}) show the same behavior at concentrations of 0.5 mM (data not shown). However, divalent cations such as Ca^{2+} and Mg^{2+} are without effect at concentrations of up to 5 mM. In addition, the presence of 5 mM Ca^{2+} or Mg^{2+} does not prevent the Zn^{2+} -dependent oligomerization.

After incubation with Zn^{2+} (1 mM for 2 h), the species with apparent molecular masses of 108 and 236 kDa were isolated by gel-filtration HPLC. Determination of the bound Zn^{2+} , using the absorbance changes due to the chelation of Zn^{2+} by 4-(2-pyridylazo)resorcinol (PAR; McCall and Fierke 2000), showed that the 236-kDa species contained an equimolar ratio of Zn^{2+} to the 55-kDa PDI monomer ($\text{Zn}^{2+}/\text{PDI} = 1.0 \pm 0.1$). This Zn -coordinated oligomer of PDI was also an active catalyst of disulfide isomerization, but it displayed only $43 \pm 6\%$ of the isomerase activity of PDI that had not been incubated with Zn^{2+} . The species with an apparent molecular mass of 108 kDa, by gel-filtration HPLC, had 0.69 ± 0.04 moles of Zn^{2+} per PDI, suggesting that both are capable of binding Zn^{2+} .

Figure 3. Time dependence of PDI oligomerization. PDI (4.4 μ M) was incubated with 1 mM Zn^{2+} for various times, and the amounts of 100-kDa species, 200-kDa species, and aggregates (>600 kDa) were determined by gel-filtration HPLC: monomer (solid circles), dimer (solid squares), and aggregate (open circles).

Although the gel-filtration results suggest that PDI (55 kDa by sequence) is a dimer that zinc converts to a tetramer, sedimentation equilibrium measurements show that the 108-kDa species is actually a monomer (Table 1) and the 236-kDa species is actually a PDI-dimer. Because gel-filtration is sensitive to the shape of the molecule, sedimentation velocity experiments were performed to evaluate the hydrodynamic properties. The sedimentation coefficients of the monomer suggest that PDI is quite elongated with an axial ratio (a/b) of 5.7. The dimer is also elongated ($a/b = 7.1$; Table 1).

The finding that the divalent cations that support PDI oligomerization are all “soft” cations with affinity for sulfur suggests that the oligomerization is dependent on PDI sulfhydryl groups. In confirmation of this suspicion, mutation of all six PDI cysteines to serines eliminates the formation of dimer and higher oligomers (Fig. 4). Replacement of the two cysteines in the **b'** domain with serines also largely

eliminates dimer formation, and replacing all four of the active site cysteines with serine shows that one or both of the PDI active sites are needed (along with those in the **b'** domain) to generate dimers and higher oligomers. Eliminating the active site cysteines of one of the catalytic domains affects the amount of dimer formed slightly but does not prevent dimerization. Thus, either of the active sites can participate in dimer formation.

Zn^{2+} is tetracoordinate, making it possible that the Zn^{2+} in the dimer is coordinated by four sulfur ligands. However, this is not the case. Mutants in which there is only one cysteine at each active site show that a single active site cysteine is sufficient to mediate oligomerization. The ligand structure of Zn^{2+} must be more complex than is its coordination of four cysteines.

In addition, to the formation of dimers, high concentrations of PDI and/or long incubation times convert wild-type PDI into large aggregates (>600 kDa; Fig. 1). The amount of PDI aggregation increases with increasing PDI concentration (Fig. 5). The presence of EDTA in excess over the Zn^{2+} concentration totally inhibits dimerization and aggregation; however, after formation of the oligomers (24 h with 0.5 mM Zn^{2+}), the addition of EDTA (5 mM) does not reverse oligomer formation substantially, even after a 3-h

Figure 4. Oligomerization of PDI mutants with cysteine to serine mutations. The amount of the PDI present as dimer after 1-h incubation with 1 mM Zn^{2+} was determined by HPLC. The reaction is not kinetically complete at this time point, and the amount of dimer formed is a complex function between aggregation and dimerization. The mutants are designated by a symbol showing the three different types of cysteine residues, including those of the CGHC sequence at the two active sites and the two located in the **b'** domain (dotted lines). An empty box indicates that both cysteines have been mutated to serine, and CX indicates that the active site cysteine nearer the C terminus (CGHC) of each active site has been mutated to ser (CGHS).

Table 1. Analytical ultracentrifugation of PDI and its oligomer

Species	Gel-filtration molecular mass (kDa)	Sedimentation equilibrium molecular mass (kDa)	Sedimentation velocity ($S_{20,w}$)	f/f_0	Axial ratio a/b^a
Monomer	108	52	3.48 ^b	1.46	5.7
Dimer	236	107	5.18 ^c	1.56	7.1

^a Prolate ellipsoid.

^b The monomeric sample also contained some dimer (30% by weight). The sedimentation velocity was identical to that observed in the dimer sample.

^c The dimeric sample also contained some (15%) higher oligomer, with an $S_{20,w}$ of 7.87.

Figure 5. PDI aggregation in the presence of Zn^{2+} . PDI at different concentrations was incubated with 1 mM Zn^{2+} at pH 7.0 (50 mM HEPES and 0.2 M NaCl) for 1 h. The relative amount of monomer (solid circles), dimer (solid squares), and aggregate (open circles) was determined by gel-filtration HPLC.

incubation (data not shown), suggesting that once the dimer is formed, the Zn^{2+} is not accessible to chelating agents. A mutant PDI that has all its active site cysteines but is missing both of the cysteines in the **b'** domain (outside the active site) also forms aggregates without forming the PDI-dimer. Thus, the formation of aggregates does not appear to be dependent on the formation of dimers.

Discussion

The heterogeneity of PDI preparations has been described in the literature on numerous occasions. Pace and Dixon (1979) first reported PDI oligomers that accumulated with storage. At least some of the oligomerization involved the formation of interchain disulfides. The presence of PDI species that exhibit heterogeneity on gel-filtration chromatography, yet show homogeneity by other criteria, has been attributed to C-terminal proteolysis (Hu and Tsou 1992) and formation of dimers (Morjana et al. 1993) or tetramers (Yu et al. 1994).

Although intermolecular disulfide formation may lead to oligomer formation in PDI, the protein can also be cross-linked through cysteines by coordination to Zn^{2+} and other divalent cations with high affinity for sulfur. Zn^{2+} at millimolar concentrations converts wild-type PDI into a dimer, which over time forms higher order oligomers and aggregates. Dimer formation and oligomerization involve cysteines. The cysteines have to be in a reduced sulfhydryl form to support oligomer formation (data not shown), and a mutant PDI with no cysteines does not form dimers or oligomers.

In addition to the four cysteines in the catalytic domains (**a** and **a'**), wild-type PDI contains two cysteines in the

noncatalytic **b'** domain. These cysteines do not contribute to PDI catalysis (Lyles and Gilbert 1994), but they are involved in dimer formation, because mutants in which these cysteines have been changed to serines do not form dimers. One active site is required for dimer formation, although it may be either one of the active sites. Eliminating all the cysteines in both active sites abolishes dimer formation. However, removing the cysteines from only one of the active sites permits dimers to form (Fig. 4). The results with mutant PDI molecules suggest that dimers can form in a variety of ways (Fig. 6), with Zn^{2+} chelated between an active site of one PDI molecule and the internal cysteines of another.

PDI dimers formed by wild-type PDI contain one atom of zinc per molecule of PDI (55 kDa) or two zinc ions per dimer. Two of the dimer models (Fig. 6, models 2 and 4) bring two pairs of interaction centers into proximity, which suggests the possibility of multiple Zn^{2+} -dependent cross links per dimer. However, we cannot exclude the possibility that there is another Zn^{2+} binding site that is independent of the sites involved in cross-linking. The observation of Zn^{2+} binding by monomeric PDI supports this view.

PDI is often described as a dimer because of its abnormal behavior on gel-filtration chromatography (Hawkins et al. 1991). Sedimentation equilibrium and velocity measurements show that PDI is a monomer in solution and that the anomalous molecular weight by gel-filtration HPLC is due to the elongated shape of the molecule. An axial ratio of 5.7 for monomeric PDI (assuming a prolate ellipsoid model) suggests that the four thioredoxin domains are arranged in a

Figure 6. Models for PDI dimerization. Dimer formation can occur through Zn^{2+} -dependent cross-linking of domains **a-b'** or domains **a'-b'**. For each type of dimer, the two PDI molecules could be oriented either in parallel or antiparallel directions. This gives rise to the possibility of four different dimers. The monomers are drawn with an axial ratio of 5.7, as suggested by sedimentation velocity experiments.

linear fashion. The thioredoxin domains **a** and **b** are approximately the same size (Kemink et al. 1996, 1999) and reasonably globular. Sequence similarity and homology modeling of the structures of the **a'** and **b'** domains suggests that they are also thioredoxin folds. Thus, PDI consists of four structural domains, all of which are likely to have thioredoxin folds. A linear arrangement of four globular domains would give an axial ratio of 4. The C-terminal tail along with the interdomain linkers could provide enough extra length to bring the axial ratio to 5.7; however, it would mean that there are few interactions between the domains of PDI. Likewise, the dimeric species is elongated, with an axial ratio of 7.1. This ratio is less than twice that of the monomer (monomer $a/b = 5.7$), suggesting that dimer formation involves some overlap of the domains, which is consistent with the models in Figure 5. The elongated shape of the dimer excludes a side-to-side alignment of the two PDI molecules and suggests that the dimer is also elongated (Fig. 5, models 1 and 4).

At high concentrations of PDI and longer incubation times, PDI forms high-molecular-weight aggregates (>600 kDa) in addition to dimers. The observation that mutation of the two cysteines in the **b'** domain eliminates dimer formation, but does not prevent the formation of aggregates, suggests that the two active sites are involved in aggregate formation and that the dimer is not an obligatory intermediate.

In proteins that incorporate zinc as a structural or catalytic cofactor, Zn^{2+} is normally coordinated by four ligands. However, four sulfur ligands are not required to bind Zn^{2+} within the PDI dimer. Mutants of PDI with only one cysteine in the active site dithiol center (CGHS) form dimers almost as efficiently as does wild-type PDI. Because only one active site cysteine is required for dimerization in these mutants, it is unlikely that the wild-type PDI dimer involves both of the cysteines in an active site. Other nearby residues, perhaps histidines, might complete the Zn^{2+} coordination.

The physiological role of the Zn^{2+} -dependent oligomerization of PDI is not yet clear. Zinc is a micronutrient widely incorporated as a structural or catalytic element in eukaryotic and prokaryotic proteins. It is predicted that the human genome encodes ~200 different versions of zinc-binding motifs, most of which contain cysteines in their zinc-binding centers (Maret 2003; Tapiero and Tew 2003). The average total Zn^{2+} concentration in whole rat liver (averaged over the whole cell volume) is in the range of 0.5 mM, $18 \pm 1\%$ of which is located in the microsomal fraction (Alfaro and Heaton 1974). Because the ER constitutes significantly <20% of the volume of the cell, Zn^{2+} would be expected to be at a relatively high total concentration in the ER. Metallothionein has been detected in the ER (Bataineh et al. 1986), suggesting that the free concentrations of Zn^{2+} might be low; however, the total concentration of metallothionein in the ER is not known, particularly in comparison

to the total concentration of Zn^{2+} . In *Schizosaccharomyces pombe*, there is a Zn^{2+} -specific transporter (Zhf) that helps sequester surplus Zn^{2+} in the ER, suggesting that ER proteins are active participants in cellular Zn^{2+} metabolism (Borrelly et al. 2002).

In addition to Zn^{2+} , PDI also binds other divalent transition metal ions, many of which are considered toxic, such as Cd^{2+} and Hg^{2+} . PDI, being a very abundant ER protein, might serve as a temporary metal ion buffer, reversibly sequestering metal ions and preventing them from binding to other proteins. However, the activity of PDI is also essential to the cell, so that the interaction of PDI with these metals might also play a role in their toxicity. In addition to its direct effects on PDI isomerase activity, Zn^{2+} , or other heavy metals in the ER, might also provide regulatory mechanisms to coordinate chaperones and folding catalysts of the ER. Baksh et al. (1995) found that in the presence of Zn^{2+} , PDI could be isolated in a complex with another ER chaperone, calreticulin.

Materials and methods

Materials

Chemicals, including 4-(2-pyridylazo) resorcinol (PAR), $ZnCl_2$, HEPES, guanidine hydrochloride, and dithiothreitol (DTT) were purchased from Sigma. Zn^{2+} acetate was obtained from Banco. P-6 Bio-gel and Chelex resins were from Bio-Rad. LB, agar, other cell culture media, and PCR reagents were from Invitrogen. PCR site-directed mutagenesis kit, DNA modification enzymes, and *Escherichia coli* strains were from Stratagene. PCR was performed by using Stratagene RoboCycler Gradient temperature cycler. Plasmid sequence verification was done on an ABI automatic sequencer. HPLC was performed by using Beckman System Gold HPLC system with a Bio-Rad DEAE-5-W column for ion-exchange chromatography and Tosohas 3000SWXL columns for gel-filtration chromatography.

PDI mutagenesis and purification

Mutagenesis to replace the "internal" cysteines of PDI, Cys294 and Cys325, with serines was accomplished by using the PCR-based Stratagene QuikChange site-directed mutagenesis kit. The plasmid pET8c containing the appropriate mutant of PDI was used as a PCR template for mutagenesis. Primers CTGAAGAAGGAGGAATCTCCAGCTGTGCGGC and GCCGCACAGCTGGAGA TTCCTCCTTCTTCAG were designed to replace the N-terminal internal cysteine. The resulting plasmid was purified and used as a template to replace the second, C-terminal, internal cysteine, using GAAGATCACACAATTTTCCCACTTCTCCTGGAG and CTCCAGGAAGTGGTGGGAAATTGTGTGATCTTC primers. The final plasmid was sequenced to verify the integrity of PDI and used for PDI expression. All PDI variants used in this article were soluble when expressed in *E. coli*.

Wild-type PDI and mutant proteins were expressed and purified essentially as described in Gilbert et al. (1991). Briefly, PDI-containing plasmids were transformed into the BL21DE3 strain of *E. coli*. At $OD_{600\text{ nm}} = 1$, cultures were induced with IPTG (0.4 mM). After 10 to 16 h, cells were isolated and disrupted by sonication. After centrifugation, the supernatant was subject to two-

stage chromatography, first on DEAE Sephacel and then on Zn-chelating Sepharose. Purified PDI was reduced, treated with EDTA, and dialyzed against 100 mM Tris-HCl buffer. The PDI was further purified by using a Bio-Rad DEAE-5-W ion-exchange column. PDI was eluted with a 0- to 0.5-M NaCl gradient over 30 min. PDI was subjected to an additional step of purification by using gel-filtration HPLC with a Tosohas 3000SWXL column (two 30 cm columns). PDI was eluted with 200 mM NaCl and 50 mM HEPES (pH 7.0) buffer.

Zn oligomer formation and HPLC experiments

HPLC-purified PDI was reduced overnight in 10 mM DTT at 4°C and centrifugally gel-filtered over a 10× volume of P-6 Bio-gel equilibrated in HPLC gel-filtration buffer (50 mM HEPES and 200 mM NaCl at pH 7.0). PDI concentration was determined by measuring OD_{280nm}, and the protein was diluted to the appropriate concentration with HPLC buffer, if necessary. PDI was incubated with the indicated Zn²⁺ concentration in 50 mM HEPES (pH 7.0; 0.2 M NaCl) at room temperature. After the indicated time, samples were subjected to gel-filtration HPLC. Samples of 20 µL were injected and eluted over 30 min (1 mL/min). Incubations of PDI-Zn samples over 24 h were performed at 4°C to prevent protein degradation.

Zn²⁺ quantitation

The concentrations of PDI and Zn were determined in fractions collected from gel-filtration HPLC. PDI was incubated with 1 mM Zn²⁺ for 2 h and subjected to HPLC. In each fraction (1 mL), the PDI concentration was determined by A280. Guanidine hydrochloride (8 M), which had been pretreated with Chelex, was added to a final concentration of 4 M, and the samples were incubated for 2 h to denature PDI. Zn²⁺ was determined by using the absorbance increase at 493 nm due to complex formation with PAR (McCall and Fierke 2000). The Zn²⁺ concentrations were determined by comparison to standards of known Zn²⁺ concentrations analyzed under the same conditions.

PDI activity assays

The isomerase activity of PDI was assayed by using reduced ribonuclease as substrate (Lyles and Gilbert 1991). Refolding was initiated by adding reduced ribonuclease (8 µM final concentration) to PDI in a glutathione redox buffer (1 mM GSH and 0.2 mM GSSG). The appearance of native ribonuclease was monitored by following the hydrolysis of the RNase substrate, cGMP, at 296 nm.

Analytical ultracentrifugation

Analytical ultracentrifugation was performed on HPLC-purified PDI monomer and Zn²⁺-induced oligomers using a Beckman XLA100 analytical ultracentrifuge. The buffer was 50 mM HEPES (pH 7.0) with 0.2 M NaCl. The protein concentration was 1.2 mg/mL. Sedimentation equilibrium was performed at 20,000 rpm for 22 h and 38 h at 20°C to ensure equilibrium. Sedimentation velocity was performed at 50,000 rpm in the same buffer with three two-fold dilutions of protein. Sedimentation coefficients (extrapolated to zero concentration) and the molecular weight were determined by using SEGAL from <http://www.biozentrum.unibas.ch/personal/jseelig/AUC/index.html>. The frictional coefficients (*f*/*f*₀) and axial ratios (*a*/*b*) were calculated by using the *v*bar method in Sednterp obtained from <http://www.jphilo.mailway.com>.

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