A Structural Disulfide of Yeast Protein-disulfide Isomerase Destabilizes the Active Site Disulfide of the N-terminal Thioredoxin Domain*

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Protein-disulfide isomerase (PDI) is an essential catalyst of disulfide formation and isomerization in the eukaryotic endoplasmic reticulum. PDI has two active sites at either end of the molecule, each containing two cysteines that facilitate thiol-disulfide exchange. In addition to its four catalytic cysteines, PDI possesses two non-active site cysteines whose location and separation distance varies by organism. In higher eukaryotes, the non-active site cysteines are located in the C-terminal half of the protein sequence and are separated by 30 amino acids. In contrast, the internal cysteines of PDI from lower eukaryotes are located near the N-terminal active site and are much closer together in sequence. The function of these cysteines and the significance of their unique location in yeast PDI have been unclear. Previous data (Xiao, R., Wilkinson, B., Solovyov, A., Winther, J. R., Holmgren, A., Lundström-Ljung, J., and Gilbert, H. F. (2004) J. Biol. Chem. 279, 49780–49786) suggest that the internal cysteines exist as a disulfide in the endoplasmic reticulum of Saccharomyces cerevisiae. By coupling mass spectrometry with a gel-shift technique that allows us to measure the redox potentials of the PDI active sites in the presence and absence of the non-active site cysteines, we find that the non-active site cysteines form a disulfide that is stable even in a very reducing environment and demonstrate that this disulfide exists to destabilize the N-terminal active site disulfide, making it a better oxidant by 18-fold. Consistent with this finding, we show that mutating the non-active site cysteines to alanines disrupts both the oxidase and isomerase activities of PDI in vitro.

Cell surface and secreted proteins are required to function in a more oxidizing environment than that of the cytoplasm and, as a result, often contain disulfide bonds that covalently link two cysteines and impart structural stability in the harsh environment of the cell exterior. In vitro, the formation of these disulfides is often slow and error-prone (1). In eukaryotes, disulfide bond formation and rearrangement occurs in the endoplasmic reticulum (ER), where it is catalyzed by protein-disulfide isomerase (PDI) (1–3), an essential, abundant, and ubiquitously expressed 55-kDa resident ER protein. Structurally, PDI is composed of four thioredoxin motifs. The rat protein exhibits a quite elongated shape in solution (axial ratio ~5.7), suggesting that the domains are extended and arranged linearly, at least in the ultracentrifuge (4). The thioredoxin domains near the N and C termini termed the a and a’ domains, respectively, contain active sites, each with a sequence CXXC that facilitates thiol-disulfide exchange. The catalytic domains are separated by two non-catalytic domains, b and b’, and the multi-domain structure terminates in a highly acidic C-terminal tail termed the c domain (abb’a’c). The b’ domain and the interface between the b’ and a’ domains contribute to peptide and protein substrate interactions (5).

In addition to its four active site cysteines, PDI contains two internal, non-active site cysteines whose function has been unclear. In mammals and birds, the internal cysteines are conserved, located in the b’ domain of the protein, and are separated by 30 amino acids. In yeast and fungi including Schizosaccharomyces pombe, the non-active site cysteines are located near the N-terminal active site in the a domain and are separated by only 6–8 residues. Luz and Lennarz (6) recently reported that mutation of the non-active site cysteines to serines in Saccharomyces cerevisiae PDI did not affect yeast growth rates; however, these cysteines were required for the efficient processing of carboxypeptidase Y (CPY), a non-essential PDI substrate whose folding, maturation, and secretion requires both the oxidase and isomerase activities of PDI, suggesting that mutation of the non-active site cysteines impaired the activity in vivo. In contrast, mutation of the non-active site cysteines of the mammalian protein has no effect on activity (7).

Recently, studies on the redox state of PDI in the yeast ER suggested that the non-active site cysteines of the a domain were present as a disulfide in vivo (2). Homology modeling of the structure of the yeast a domain predicts that the non-active site cysteines are within the covalent bonding distance of each other, suggesting a structural role. Using a gel-shift assay to count the number of free sulfhydryl groups under various redox conditions and mass spectrometry to identify the redox state of the non-active site cysteines, we find that the non-active site cysteine pair forms an extremely stable disulfide whose reduction is only efficiently effected by a strong reducing agent such as DTT. In contrast to the mammalian protein (8) in which the

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¶ The abbreviations used are: ER, endoplasmic reticulum; DTT, dithiothreitol; Mal-PEG, maleimide-conjugated polyethylene glycol polymer; NEM, N-ethylmaleimide; PDI, protein-disulfide isomerase; MALDI, matrix-assisted laser desorption ionization; CPY, carboxypeptidase Y; $K_a$, equilibrium constant.
Disulfides in Yeast PDI

EXPERIMENTAL PROCEDURES

Materials—GSH, GSSG, DTT, N-ethylmaleimide (NEM), and trichloroacetic acid were obtained from Sigma. PCR primers were ordered from Integrated DNA Technologies (Skokie, IL). DNA polymerase, T4 DNA ligase, and restriction enzymes were obtained from Invitrogen.

Site-directed Mutagenesis and Purification of PDI from Escherichia coli—The coding sequence of yeast PDI was PCR-amplified from pCT-38 (9) to contain a 5′-Ndel site and a 3′-XhoI site. After digestion with NdeI and XhoI, the PCR products were inserted between the corresponding sites of the pET23a vector (Novagen) to create plasmid pET23a-YPDI, which adds a C-terminal His tag to the mature sequence of yeast PDI. A PDI mutant with the two non-active site cysteines mutated to alanines, C90A,C97A yeast PDI (numbering starting with the coding sequence of the yeast enzyme, GenBank™ accession number NP_009887) was generated using the QuikChange site-directed mutagenesis kit from Stratagene. A pET23a-YPDI template was used along with appropriate mutagenic primers (5′-ACT GAA AAC CAG GAT CT GGC TG TGT CCG TTG GCA TAC ATC T G CAGA TCC TGC GTG TCG ATT CTT TGG CAC CTA GC CAC CCA GAC CAT CT GTG GCG CCA GAT CCT TG TGT GTT CTA ga aac cag gat ct ggc tgg tgt cgg tca tgg ctc tgc ggt cct tga ggt ttt cag ta gct gaa cag(gc) to generate plasmid pET23a plasmid to generate pET23a-C90A,C97A yeast PDI. The sequences of the coding regions of the wild-type and mutant PDI genes were confirmed by DNA sequencing.

E. coli strain BL21 (Gold) DE3 (Invitrogen) transformed with the appropriate pET23a vector containing either wild-type or mutant yeast PDI was grown at 37 °C in LB medium supplemented with 100 μg/ml ampicillin to an absorbance of 1.0 at 600 nm and induced by adding isopropyl β-D-thiogalactoside to a final concentration of 1 mM. After 4 h, the cells were harvested by centrifugation, resuspended in ice-cold loading buffer (pH 7.5, 20 mM phosphate, 0.5 mM NaCl, and 100 mM imidazole), and disrupted by sonication. After centrifugation at 12,000 × g for 15 min at 4 °C, the supernatant was applied to a HiTrap chelating column (Amersham Biosciences) precharged with nickel and equilibrated with loading buffer. The column was washed with 10 ml of loading buffer and then with 5 ml of elution buffer containing 300 mM imidazole. Eluate fractions containing PDI were pooled and dialyzed against 50 mM Tris-HCl, 1 mM EDTA, pH 8.0 to remove the imidazole. The purity of proteins was analyzed by SDS-PAGE and estimated to be ≥98%.

Redox Potential Measurements—Maleimide-conjugated polyethylene glycol (Mal-PEG) was obtained from Nektar Therapeutics (San Carlos, CA) and was purified before use by gel filtration on a PD-10 column (Amersham Biosciences) to remove low-molecular weight maleimides. These lower molecular weight maleimides may react with free sulfhydryl groups but do not significantly increase the molecular weight, leading to incomplete shifts of the protein on SDS-PAGE in fully reduced controls (2). Wild-type or mutant PDI (3.5 μM) was incubated in various GSH/GSSG-disulfide redox buffers in 0.1 M Tris, pH 8.0, for 30 min at room temperature. Protein was precipitated by the addition of an equal volume of ice-cold 40% trichloroacetic acid (20% final concentration). The protein was resuspended in 5 mM Mal-PEG in non-reducing 2× SDS sample buffer (3% SDS, 0.2 μM Tris-HCl, pH 8, glycerol, and bromophenol blue), incubated for 30 min at room temperature, and then quenched by the addition of DTT to a final concentration of 50 mM.

Results—Reduced and oxidized controls were prepared by incubating the proteins in either 10 mM DTT in 2× SDS sample buffer or 1 mM GSSG in 0.1 M Tris, pH 8.0, for 30 min at room temperature, acid-precipitating the protein with trichloroacetic acid (20% final concentration), resuspending in Mal-PEG, and then quenching by the addition of DTT to a final concentration of 50 mM.

Samples were resolved by SDS-PAGE on precast 4–20% Tris-HCl gels (Bio-Rad) and visualized by Coomassie Blue staining. Band intensities were determined after subtracting a constant background from the images using Scion Image software (www.scioncorp.com). The fraction of the total cysteines present as sulphydryl groups was determined by multiplying the intensity of each band times the number of sulphydryl groups represented in the band, summing, and dividing by the total intensity. Redox potentials were calculated by plotting the total number of free sulphydryl groups versus [GSH]2/[GSSG]. Kak represents the equilibrium constant for the reaction shown in Equation 1.

\[
\text{SH} + \text{S} = \text{SH} + \text{S} \quad \text{Equation 1}
\]

The data were fit by non-linear least squares to a model (Equation 2) that contained a single intramolecular disulfide in the oxidized state and two independently titrating sites where each site contained two sulphydryl groups in the reduced state.

\[
\text{Total SH} = 2 \times \frac{[\text{GSH}^2][\text{GSSG}]}{[\text{GSH}^2][\text{GSSG}] + K_{\text{eq}}} = 2 \times \frac{[\text{GSH}^2][\text{GSSG}]}{[\text{GSH}^2][\text{GSSG}] + K_{\text{eq}}} \quad \text{Equation 2}
\]

For both the wild-type and mutant proteins, the fit of the data to a two-site model was significantly better than the fit of the data to a one-site model (10).

Mass Spectrometry—Wild-type and mutant proteins were prepared in the fully oxidized form by incubation with 1 mM GSSG. Protein with two sites reduced was prepared by equilibration with 8 mM GSH plus 0.2 mM GSSG. The fully reduced protein was generated using 10 mM DTT. All of the incubations were performed in 0.1 M Tris, pH 8.0, for 30 min at room temperature. Protein was precipitated by the addition of an equal volume of ice-cold 40% trichloroacetic acid (20% final concentration) and resuspended in 20 mM NEM in 0.1 M Tris, pH 8.0, and incubated for 1 h at room temperature. Unreacted NEM was removed by centrifugal gel filtration through Sephadex G-50 (Amersham Biosciences) that had been swelled and prewashed in 50 mM Tris, pH 8. DPD samples (10 μg) then were digested with 1 μg of Lys-C (WAKO) in 50 mM Tris, pH 8, for 20 h at 37 °C and desalted using a ZipTip. Peptides were eluted from the ZipTip with 3–6 μl of an aqueous solution of 60% methanol and 2% formic acid. 1 μl was spotted on a MALDI target plate, dried, and matrix-spotted (cyano-4-hydroxycinnamic acid) and dried following analysis in reflector mode on an Applied Biosystems Voyager DE-STR MALDI-time-of-flight mass spectrometer. Monoisotopic peptide masses detected were sent to ProFound (PROWL, Rockefeller University) or MS-FIT (Protein Prospector, University of California, San Francisco) for protein data base searches and protein identification by peptide mass fingerprinting.

Ribonuclease Refolding Assay—Renaturation of reduced RNase was followed in a continuous assay as described previously (11). The formation of active RNase was measured spectrophotometrically by monitoring hydrolysis of the RNase substrate cCMP at 296 nm. Each sample contained 4.5 mM cCMP, 1 mM GSH, 0.2 mM GSSG, 100 mM Tris-HCl, pH 8.0, 1 mM EDTA, 8 μM reduced RNase, and 0–9 μM wild-type or mutant yeast PDI. The assay was performed at 25 °C and initiated by the addition of reduced RNase.

The isomerase activity was determined from the linear increase in the amount of enzymatically active RNase with time after a short lag, which reflects the oxidase activity. The lag time was determined from the x-intercept of a plot of RNase activity against time. The oxidase activity was estimated evaluated from linear plots of 1/ΔM against time and is reported as the slope of this plot in units of μM−1 min−1.

RESULTS

Previous observations of the redox states of yeast PDI in the yeast ER suggest that the non-active site cysteines are present as a disulfide (2). This suggestion is strengthened by homology modeling the sequence of the yeast PDI-a domain onto the NMR structure of the human PDI-a domain with 3D-PSM (www.sbg.bio.ic.ac.uk) (Fig. 1). The homology model suggests that the two cysteines outside the active site are sufficiently close to form a disulfide. In the fully oxidized state produced by incubating the wild-type protein with 1 mM GSSG, there are no free cysteines in the molecule, consistent with the idea that all of the cysteines in the protein are present as disulfides.
Redox Potential Measurements—The three disulfide bonds of yeast PDI have different stabilities as indicated by measurements of the number of free sulfhydryl groups after equilibration with different glutathione redox buffers. A gel-shift assay that adds a polyethylene glycol-conjugated maleimide to each sulfhydryl group (12) was used to count the number of free sulfhydryl groups present in both wild-type yeast PDI and a mutant of yeast PDI in which the non-active site cysteines are mutated to alanines (C90A,C97A). Mal-PEG alkylation of a single sulfhydryl results in an apparent molecular mass shift of ~15 kDa as observed by SDS-PAGE. The number of 15-kDa shift increments of PDI on a gel indicates the number of cysteines that are present as sulfhydryl groups, because cysteines that are participating in disulfide bonds will not shift upon Mal-PEG treatment.

As seen in Fig. 2, equilibration of the wild-type protein with an increasingly more reducing glutathione redox buffer leads to the availability of additional sulfhydryl groups that can be modified with Mal-PEG. In the fully oxidized state (lane 1) produced by incubating the protein with 1 mM GSSG, no Mal-PEG adducts were formed. In a series of glutathione redox buffers, the predominant bands observed for the wild-type protein occurred at two and four free sulfhydryl groups, suggesting minimal formation of glutathione-mixed disulfides involving only one sulfhydryl group. At the highest [GSH]/[GSSG] examined (380 mM), only four sulfhydryl groups could be modified. However, complete reduction with DTT (10 mM) resulted in six Mal-PEG additions, indicating that one of the three cysteine pairs forms a very stable disulfide that can only be reduced with DTT.

The redox potential of a cysteine pair can be expressed as the equilibrium constant ($K_{ox}$) for the oxidation of that cysteine pair by GSSG, which produces two molecules of GSH (Equation 1). If the redox potentials are sufficiently different, the three disulfides can be selectively reduced. Equilibration in a number of different redox buffers of differing [GSH]/[GSSG] followed by acid quenching, removal of the redox buffer, and modification with a gel-shift sulfhydryl reagent, Mal-PEG, displays the redox state of the protein as a function of the redox buffer composition (Fig. 2). The number of sulfhydryl groups was determined by integrating the intensities of the bands on the stained gel after Mal-PEG modification. For both the wild-type and mutant proteins, equilibration with different redox buffers shows a broad titration of two disulfides (four sulfhydryl groups) (Fig 3) as the value of [GSH]/[GSSG] increases. For the wild-type protein, DTT (10 mM) is needed to reduce the remaining disulfide, but for the C90A,C97A mutant protein, DTT reduction does not reveal an additional disulfide.

The redox potentials of the individual sites was determined by fitting the number of free sulfhydryl groups observed by Mal-PEG alkylation to a model (Equation 2) in which two disulfides equilibrate independently. DTT controls were omitted from the statistical analysis but are shown in Fig. 3 to indicate the residual disulfide that can be reduced only with DTT. Both the wild-type and non-active site mutant proteins show a biphasic titration of two disulfides as the redox buffer becomes more reducing. Non-linear least squares fitting provides estimates of the thiol-disulfide oxidation potentials ($K_{ox}$) for each site (Table 1). For the wild-type protein, the $K_{ox}$ values are $1.0 \pm 0.2$ mM and $17 \pm 3$ mM and a third disulfide is sufficiently stable to resist reduction by glutathione. Because $<10–20\%$ of the most stable disulfide could be reduced by glutathione, we can estimate that the $K_{ox}$ value for its formation is greater than 1600 mM. When the non-active site cysteines are mutated to alanines, all of the disulfides of the mutant protein can be reduced by glutathione. The measured redox potentials for the disulfide that is reduced at the lowest [GSH]/[GSSG] are identical for the wild-type and mutant proteins. However, the $K_{ox}$ of disulfide that titrates next in the mutant protein is ~20-fold higher (more difficult to reduce) than the disulfide that titrates second in the wild-type protein. Consequently, two titratable disulfides in wild-type PDI could correspond to the two active sites or they could correspond to one active site and the non-active site disulfide, depending on which cysteine pair forms the most stable disulfide.

The Non-active Site Cysteines of Yeast PDI Form a Structural Disulfide—To determine which of the cysteines form a disulfide that cannot be reduced with glutathione, wild-type yeast PDI was incubated with GSSG (1 mM) to yield a species that did not
react with Mal-PEG (no shift). Digestion of the GSSG-oxidized protein with Lys-C and MALDI mass spectrometry revealed a peptide of mass 1764.81 Da corresponding to the peptide containing the active site cysteines of the PDI-a domain and a peptide of mass 3143.4 Da corresponding to the peptide expected from a disulfide between the non-active site cysteines. The larger peptide containing the PDI-a domain active site cysteines (expected monoisotopic mass of disulfide containing peptide is 3375.5) was not observed, suggesting that its ionization was suppressed under the conditions of the MALDI experiment. Wild-type yeast PDI was partially reduced with a glutathione reduct buffer (8 mM GSH and 0.2 mM GSSG) so that there were four PDI sulfhydryl groups. After NEM alkylation to prevent thiol/disulfide rearrangements, Lys-C peptide maps show that the peptide containing the non-active site cysteines (3143.4 Da) is still present. Upon full reduction of the wild-type protein with DTT, the peptide containing the non-active disulfide (3143.4 Da) disappears, indicating that it is the non-active site disulfide that is resistant to glutathione reduction.

**Enzymatic Activity**—Mutation of the non-active site cysteines to alanine also has a significant effect on the oxidase and isomerase activities of yeast PDI using reduced RNase A as substrate. During the oxidative folding of reduced RNase A, the oxidase activity of PDI converts the reduced RNase A to a complex mixture of inactive oxidized forms that then are converted to native RNase A by the disulfide isomerase activity (13). The oxidase activity is indicated by the lag time for the appearance of active RNase after initiation of the refolding, whereas the isomerase activity can be measured after the lag by the appearance of enzymatically active RNase A. Under these conditions, both the oxidase and isomerase activities of the mutant PDI are compromised (Table II). Thus, the integrity of the non-active site cysteines of the PDI-a domain is needed for full oxidase and isomerase activity.

**DISCUSSION**

PDI is a highly conserved protein. The vertebrate enzyme is 96% conserved from chicken to human (86% identical, 96% similar), and even the yeast and mammalian proteins share 38% identity in the catalytic domains. Along with the overall domain architecture in which four thioredoxin domains are linked together, the sequences around the active site cysteines are strictly conserved among all of the eukaryotes including yeast and fungi (Fig. 4). In vertebrate PDIs, including those of human and chicken, there are also two conserved cysteines outside the active site but they are located in the non-catalytic PDI-b domain. Their mutation to serine has no effect on enzymatic activity (7), and the two non-active site cysteines appear to be somewhat shielded from solvent (5). When expressed as a heterologous protein in the yeast ER (2) and in vitro under optimum redox conditions, the two non-active site cysteines of the vertebrate protein are present as sulfhydryl groups. In yeast and fungal PDIs, including that of Dictyostelium discoideum, there are also two conserved cysteines outside the active site; however, in these organisms, they are conserved within the catalytic PDI-a domain. In the yeast ER, approximately two of the six cysteines of yeast PDI are present as sulfhydryls. Mutation of the non-active site disulfide does not appreciably change the number of free cysteines, suggesting that the non-active site cysteines are present as a disulfide in vivo (2). Westphal et al. (14) observed that mutating all of the catalytic active site cysteines to serines in the yeast PDII and PDIII domains destroyed the catalytic activity, suggesting that the role of the remaining non-active site cysteines is structural rather than catalytic.

Luz and Lennarz (6) investigated the contribution of the non-active site cysteines to the function of S. cerevisiae PDI in vivo by mutating them to serine. Although the non-active site cysteines of PDI were not essential for cell growth and viability, they were required for the efficient processing of CPY (6). Expression of the yeast PDII-a domain supports normal doubling times and CPY processing in the yeast ER (2). This implies that mutation of the structural disulfides in the a domain has a more significant effect on protein maturation in vivo.

**Fig. 4.** PDI-a domain alignment. PDI sequences from various organisms were aligned in T-coffee (www.ch.embnet.org/software/TCoffee.html) and shaded using BOXSHADE. Identical sequences are shown with white text and highlighted in dark gray. Conserved sequences are shown with black text and highlighted in light gray. Cysteines are highlighted in black and indicated with arrows.

**Table I**

| Thiol/disulfide redox potentials (K_m) for wild-type and mutant yeast PDI |
|-----------------|-----------------|
| PDI             | K_m [mM]        |
| Wild type       | 1.0 ± 0.2       |
| C90A,C97A       | 2.0 ± 0.4       |

**Table II**

| Oxidase activities of wild-type and mutant yeast PDI |
|-----------------------------------------|-----------------|-----------------|------------------|
| PDI | Oxidase activity [μM⁻¹ min⁻¹] | WT PDI | Disulfide⁻⁻ isomerization | WT PDI |
|-----|-------------------------------|--------|----------------------------|--------|
| Wild type | 6.3 ± 0.6 | 100 | 0.26 ± 0.035 | 100 |
| C90A,C97A | 2.3 ± 0.3 | 36 | 0.05 ± 0.02 | 19 |

a Assays were performed using 8 μM rRNase in 4.5 mM cCMP, 100 mM Tris-HCl, and 2 mM EDTA, pH 8.0, and 25 °C in a glutathione reduct buffer (1 mM GSH, 0.2 mM GSSG).

b The oxidase activity is reflected by the decrease in the lag time preceding the formation of native RNase from reduced RNase as the PDI concentration increases. Calculation of the oxidase activity is described under “Experimental Procedures.” Calculations were restricted to PDI concentrations where the activity is linear with protein concentration.

The isomerase activity is the linear rate of appearance of catalytically active RNase A from reduced RNase measured after the initial lag. Calculations were restricted to PDI concentrations where the activity is linear with protein concentration.
the ER than deletion of three domains of the protein, including the a domain. The results of our in vitro activity assays (Table II) confirm that mutation of the structural disulfides of the yeast protein has a very significant effect on both the oxidase and isomerase activities, decreasing the oxidase activity to 36% wild-type protein and decreasing the isomerase activity to only 20% wild-type protein. The effects of mutating the structural disulfide of the a domain are more significant on catalytic activity than mutating the catalytic cysteines of the a domain. Westphal et al. (14) found that a yeast PDI mutant with the catalytic cysteines replaced by serine had 50% oxidase activity of the wild-type protein and 50% isomerase activity.

In contrast to the vertebrate protein whose two active sites have indistinguishable redox potentials of 1–2 mM (8, 7), the active sites of the yeast protein titrate with independently measurable redox potentials. The most easily reduced disulfide in the yeast enzyme has the same oxidation potential \( K_{ox} \) in both the mutant and wild-type protein, suggesting that it represents the redox potential of the a’ active site. Consequently, the \( K_{ox} \) of the a’ domain of the yeast protein (1 mM) is quite similar to that of the active sites of the mammalian protein.

Because partial reduction of the wild-type protein does not reduce the disulfide between the non-active site cysteines, the second disulfide to titrate in the redox titration of the wild-type protein must be the active site disulfide of the a domain. The \( K_{ox} \) of the second disulfide to titrate is 17 mM. The active site disulfide of the a domain is almost 20-fold more difficult to reduce than the disulfide of the a’ domain \( (K_{ox} = 1 \text{ mM}) \). Thus, the disulfide formed at the active site of the yeast PDI-a domain is structurally more stable than the disulfide formed at the a’ domain by a difference that amounts to ~1.6 kcal/mol.

Surprisingly, when the non-catalytic structural disulfide in the a domain is destroyed by mutation, the active site disulfide of the a domain becomes significantly more stable. The \( K_{ox} \) for reduction of this disulfide increases from 17 mM in the wild-type protein to ~300 mM in the mutant protein, an 18-fold increase in disulfide stability in the mutant protein. This implies that the role of the structural disulfide in PDI from lower eukaryotes is to destabilize the disulfide of the a domain, making it a better oxidizing agent thermodynamically.

The finding that the active sites have significantly different oxidation potentials may have implications for PDI catalysis in vivo. In the yeast ER, PDI has on average ~1.4 free active site sulfhydryl groups/molecule (2). Tsai and Rapoport (15) have reported that the a’ active site is preferentially oxidized by Ero1 in the yeast ER, implying that in vivo the a domain active site cysteines are present as sulfhydryls, whereas the a’ domain active site cysteines form a disulfide. It is not unreasonable then to predict that in yeast, disulfide formation, which requires an oxidized PDI active site, is performed primarily by the a’ domain active site, whereas disulfide isomerization, which requires a reduced active site, is performed primarily by the a domain active site. Consistent with this speculation is the finding by Westphal et al. (14) that the a domain is more important than the a’ domain for the maturation of CPY in vivo.

Because the oxidase activity of PDI is more essential for yeast viability than its isomerase activity (2), disrupting the domain responsible primarily for isomerization should have no effect on cell growth and viability. Luz and Lennarz (6) mutated the non-active site cysteines of yeast PDI and found no consequences on cell growth and viability (6). However, they did observe that CPY folding was impaired. In the absence of the non-active site disulfide, the a domain active site disulfide is stabilized 18-fold. Therefore, the a domain active site is 18 times more difficult to reduce in a mutant PDI lacking the non-active site cysteines than in the wild-type protein. The inability of the a domain active site of this mutant to be reduced under in vivo redox conditions and to catalyze disulfide isomerization could account for the impairment in CPY folding observed by Luz and Lennarz (6).

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A Structural Disulfide of Yeast Protein-disulfide Isomerase Destabilizes the Active Site Disulfide of the N-terminal Thioredoxin Domain
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