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Domain Architecture of Protein-disulfide Isomerase Facilitates Its Dual Role as an Oxidase and an Isomerase in Ero1p-mediated Disulfide Formation*♦

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Native disulfide bond formation in eukaryotes is dependent on protein-disulfide isomerase (PDI) and its homologs, which contain varying combinations of catalytically active and inactive thioredoxin domains. However, the specific contribution of PDI to the formation of new disulfides *versus* reduction/rearrangement of non-native disulfides is poorly understood. We analyzed the role of individual PDI domains in disulfide bond formation in a reaction driven by their natural oxidant, Ero1p. We found that Ero1p oxidizes the isolated PDI catalytic thioredoxin domains, A and A′ at the same rate. In contrast, we found that in the context of full-length PDI, there is an asymmetry in the rate of oxidation of the two active sites. This asymmetry is the result of a dual effect: an enhanced rate of oxidation of the second catalytic (A′) domain and the substrate-mediated inhibition of oxidation of the first catalytic (A) domain. The specific order of thioredoxin domains in PDI is important in establishing the asymmetry in the rate of oxidation of the two active sites thus allowing A and A′, two thioredoxin domains that are similar in sequence and structure, to serve opposing functional roles as a disulfide isomerase and disulfide oxidase, respectively. These findings reveal how native disulfide folding is accomplished in the endoplasmic reticulum and provide a context for understanding the proliferation of PDI homologs with combinatorial arrangements of thioredoxin domains.

Proteins that traverse the secretory pathway typically contain disulfide bonds that are critical for their correct fold and function. In eukaryotes, the endoplasmic reticulum (ER)[2] is the entry point into the secretory pathway and is the cellular compartment where folding and disulfide bond formation occur (1, 2). Disulfides can form spontaneously *in vitro* in the presence of an oxidizing agent such as molecular oxygen or oxidized glutathione; however, this process is typically slow and inefficient. *In vivo*, disulfide bond formation is dependent on cellular machinery to catalyze the formation of new disulfides (oxidation) and the rearrangement of non-native disulfides (isomerization). Both oxidation and isomerization are necessary for allowing the full complement of native disulfide bond formation.

Protein-disulfide isomerase (PDI), which was identified more than 40 years ago, plays a critical role in promoting native disulfide bond formation *in vivo* (3). PDI, an essential enzyme with the ability to catalyze both the oxidation of new disulfides and the isomerization of existing disulfides, is composed of four thioredoxin-like domains (4). The first and last domains (referred to as A and A′, respectively) contain Cys-*x*-*x*-Cys (C*xx*C) active sites, whereas the two middle domains (referred to as B and B′) are catalytically inactive (5, 6). Oxidation involves the transfer of an active site disulfide from PDI to substrate proteins, while isomerization requires the active site cysteines to be in a reduced form so that they can attack non-native disulfides in substrate proteins thereby catalyzing their rearrangement. Thus, oxidation and isomerization have opposing requirements for the redox state of PDI.

Analyses of the cellular role of PDI have led to a conflicting picture about whether it functions primarily as an isomerase or an oxidase. In *Saccharomyces cerevisiae*, a PDI mutant in which the second active-site cysteine in both of the catalytic domains is replaced with serine (C*xx*S-C*xx*S) is able to restore viability to a Δ*pdi1* strain (7). Since isomerization only requires one cysteine per active site, this implies that the isomerase activity of PDI is essential. However, in *S. cerevisiae*, the inactivation of PDI leads to a dramatic defect in the *de novo* formation of disulfide bonds in newly synthesized proteins (8). This suggests that PDI also plays an important role in catalyzing disulfide oxidation.

The picture is further complicated by the existence of four other yeast PDI homologs in the ER: Mpd1p, Mpd2p, Eug1p, and Eps1p (9–12). The PDI family is even larger in metazoans and now includes at least 17 different human proteins (13). These homologs possess varying combinations of catalytic and non-catalytic thioredoxin domains as well as additional trans-membrane and chaperone domains. In general, it is poorly understood what contribution these complicated domain architectures make given that the thioredoxin domain is the basic catalytic unit. One possibility is that the specific domain combinations dictate the interactions between a PDI homolog and other ER-resident chaperones to define a subset of folding substrates. A well studied example of this is the human protein, ERp57, which acts in concert with the ER lectin chaperones, calreticulin and calnexin, to specifically aid in the folding of glycosylated substrates (14–16).

Another possibility is that the different domain combinations may dictate distinct redox functions for the PDI homologs. Many studies have explored the functional differences among PDI homologs by examining the redox properties of the different thioredoxin catalytic domains (reviewed in Ref. 1). These studies have typically focused on measurements of redox potentials using small molecule thiol agents such as glutathione. The redox potential of a thioredoxin domain can be used successfully to distinguish between the more strongly reducing cytosolic thioredoxin domains and the ER localized PDI family members (17). However, recent studies have shown that disulfide bond for-

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[2] The abbreviations used are: ER, endoplasmic reticulum; PDI, protein-disulfide isomerase; GuHCl, guanidine hydrochloride; AMS, 4-acetamido-4′-maleimidylstilbene-2,2′ disulfonic acid; CPY, carboxypeptidase Y.

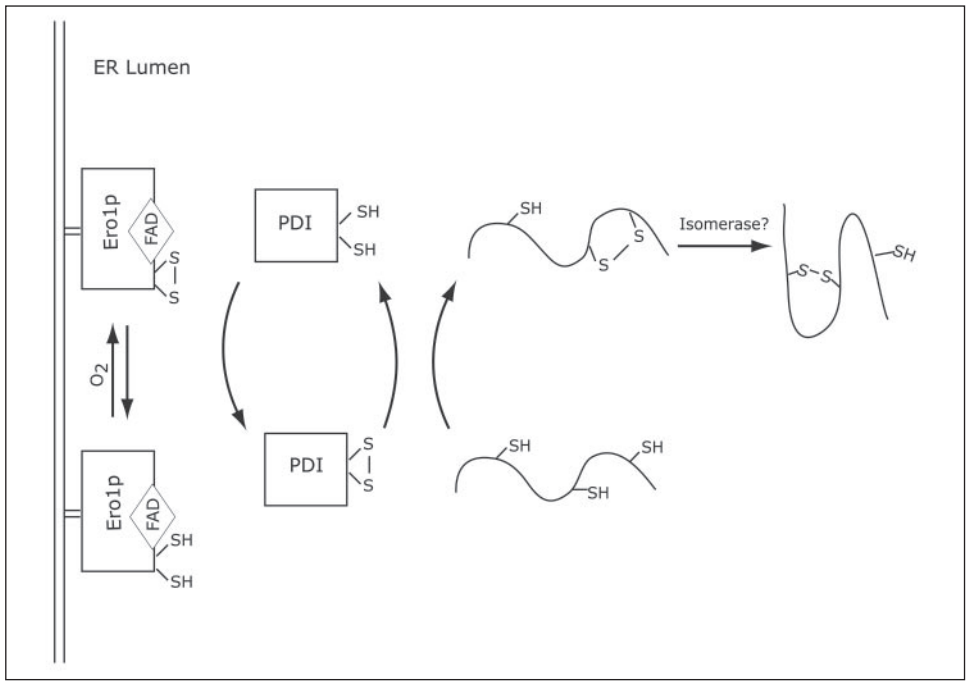

FIGURE 1. **A schematic model of oxidative protein folding in the ER.** Disulfide bonds are formed in substrate proteins by thiol-disulfide exchange with oxidized PDI. Disulfides in PDI are regenerated by interaction with Ero1p, a FAD-bound protein. Ero1p is oxidized by molecular oxygen under aerobic conditions and an undefined electron acceptor under anaerobic conditions (25). The mechanism of disulfide isomerization is the subject of the present study.

mation occurs independently of glutathione both *in vivo* (18) and *in vitro* (19). *In vivo*, the redox state of PDI is determined by its interaction with Ero1p rather than by equilibrium with the bulk glutathione redox buffer in the ER (8). Ero1p is an essential and evolutionarily conserved protein that generates disulfides in a flavin-dependent reaction that consumes molecular oxygen (Fig. 1) (19–24). Ero1p does not transfer oxidizing equivalents directly to folding substrates. Instead, Ero1p specifically oxidizes PDI which in turn passes the oxidizing equivalents to substrates (Fig. 1) (8, 19). Thus, to understand the specific cellular role of PDI and its homologs, we have to establish whether and how these proteins interact with Ero1p.

In the present study, we systematically explore the contribution of PDI to native disulfide bond formation in a reaction driven by Ero1p. Taking advantage of the reconstitution of the Ero1p pathway (19), we show that there is an asymmetry in the rate of oxidation of the two PDI catalytic domains that allows it to function as a dual disulfide oxidase/isomerase. We also demonstrate that this asymmetry results from a combination of two effects: first, the enhanced rate of oxidation of the A′ domain in the context of the full-length protein and second, the substrate-mediated inhibition of oxidation of the A domain. We further establish that the asymmetry is not intrinsic to the individual catalytic domains and rather the specific order of thioredoxin domains in PDI is critical for the functional asymmetry in its active sites.

EXPERIMENTAL PROCEDURES

Plasmid Construction and Protein Purification—Plasmids for bacterial expression of yeast PDI and $A_{AxxA}A'_{CxxC}$ were published previously (19, 25). $A_{CxxC}A'_{AxxA}$ was generated by the QuikChange site-directed mutagenesis (Stratagene) of the second active site of the PDI expression vector pBT101. PDI fragments BB′A′, B′A′, A′, A, and AB were generated by PCR and cloned into pBT101 backbone using XmaI and XhoI. The primers used to generate the fragments are as follows: PDI-5′BB′A′, TATTACCCGGGGCATGATCAAGCAAAGCCAACCG; PDI-5′B′A′, TATTACCCGGGGCAAAGCCGATATCGCTGACGCTG; PDI-5′A′, TATTACCCGGGGCGATGCCTCCCCAATCGTGAAGTCCCAAG; PDI-3′ for all of the above, GATTACTCGAGCTTACAATTCATCGTGAATGGCATC; PDI-3′A, GTGGTCGAACCGATTACTCGAGCTTATTGCTTGATCATGAATTGGAC; PDI-3′AB, GATTACTCGAGCTTACTTACCGTTGTATACTACAGG; PDI-5′AB and A, TATTACCCGGGGCGCCCCTGAAGACTCCGCTGTCG.

All of the generated plasmids were verified by DNA sequencing. All the constructs are N-terminally His tagged and were purified over a nickel column as described previously (19). Ero1p was purified as described previously (19). The concentration of PDI was determined by A_{280} in 6 M GuHCl, and the concentration of Ero1p was determined using a Coomassie-based assay (Bio-Rad). Full-length ERp57, ERp57 A domain, and ERp57 A′ domain were purified as described previously (26).

RNase A Re-oxidation Assay—Reduced RNase A was prepared as described previously (19). The folding reactions were initiated by addition of reduced RNase A and reduced PDI or ERp57 to Ero1p and 20 μM FAD in a final reaction buffer (100 mM Tris acetate (pH 8.0), 50 mM NaCl, and 1 mM EDTA). At the indicated time points, free thiols were blocked by the addition of SDS loading buffer and 10 mM AMS (4-acetamido-4′-maleimidylstilbene-2,2′ disulfonic acid, Molecular Probes). The oxidized and reduced forms of RNase A were detected by Coomassie staining after separation by non-reducing SDS-PAGE.

RNase A Activity Assay—RNase A activity was assayed by the hydrolysis of cCMP (Sigma) (19). Reduced RNase A (20 μM) was added to a reaction containing the indicated concentrations of Ero1p, reduced PDI or ERp57, FAD (100 μm), and cCMP (18 mM) in a buffer containing 100 mM Tris acetate (pH 8.0), 50 mM NaCl, 1 mM EDTA, and 0.005% digitonin. RNase A activity was monitored by the change in absorbance at 296 nm at 25 °C using an absorbance plate reader (Molecular Devices).

Fluorescence Assay of PDI Oxidation—Oxidation of PDI was monitored using the dye ThioGlo1 (Covalent Associates, Inc.). ThioGlo1 is a multimode derivative of naphthopyranone that has little background fluorescence (27). It reacts rapidly with free thiols to give a fluorescent product and can be used to detect protein thiol concentrations as low as 10 nM (27). The reactions are monitored using a fluorescence plate reader (Molecular Devices) with the excitation wavelength set at 379

nm and the emission measured at 510 nm. PDI was reduced using 10 mM dithiothreitol and buffer exchanged using a Centri-Sep spun column (Princeton Separations) into 100 mM Tris acetate (pH 8.0), 50 mM NaCl, and 1 mM EDTA. Reactions were started by the addition of reduced PDI to the indicated concentration of Ero1p and FAD (20 μM). For measurements of the oxidation rate in the presence of RNase A, reactions were started by the addition of reduced PDI and reduced RNase A to Ero1p and FAD (20 μM) at the indicated concentrations. The final reaction buffer contained 100 mM Tris acetate (pH 8.0), 50 mM NaCl, and 1 mM EDTA. The disulfide content was analyzed at the indicated time points by the addition of 6 M GuHCl, 1× phosphate-buffered saline, and 25 μM ThioGlo1. The samples were incubated for 10 min and analyzed by fluorescence. The initial rate of oxidation was calculated as the slope of the linear decrease in fluorescence over time. The assay with ERp57 was done following the same protocol.

RNase A Protection Assay—To prepare reduced and alkylated RNase A, 10 mg of protein was reduced in 1 ml of 6 M GuHCl, 0.1 M Tris-HCl (pH 8.0) and 10 mM DTT. RNase A was reduced for 30 min at 37 °C and iodoacetamide (Sigma) was added to a final concentration of 80 mM. The protein was incubated in the dark for another 30 min at 37 °C. The sample was then desalted using a Centri-Sep spin column into 20 mM Hepes (pH 7.0) and 50 mM NaCl and protein concentration was determined by A_{280} in 6 M GuHCl. Blocked RNase A (200 μM) was added to the reaction mixture with reduced PDI or ERp57, Ero1p (0.3 μM), and FAD (20 μM). The rate of oxidation was compared in the presence or absence of alkylated RNase A.

RESULTS

Both the PDI Active Site Mutants Have Large Defects in Promoting RNase A Refolding—The individual contributions of the two yeast PDI active sites to Ero1p-mediated disulfide bond formation are poorly characterized. To dissect their roles, we made two active site mutants in which the pair of active site cysteines in the first ($A_{AxxA}A'_{CxxC}$) and last ($A_{CxxC}A'_{AxxA}$) thioredoxin domains was mutated to a pair of alanines (Fig. 2*A*). We characterized the activity of these mutants by reconstituting the complete Ero1p pathway using purified Ero1p, PDI, FAD, and the folding substrate RNase A (19). RNase A is a well characterized substrate that depends on the correct formation of four disulfide bonds for folding and activity (28). We followed native disulfide bond formation in refolding RNase A by monitoring its activity using the artificial substrate cCMP. We found that both $A_{AxxA}A'_{CxxC}$ and $A_{CxxC}A'_{AxxA}$ have large defects in promoting RNase A folding when compared with wild-type PDI under conditions in which the number of active sites are matched (Fig. 2*B*).

In principle, the discrepancies in the abilities of the active site mutants to refold RNase A could result from a defect in either disulfide oxidation or isomerization. To help differentiate between these possibilities, we directly tested the ability of the two active site mutants to support disulfide formation by using an assay that, unlike the activity assay, does not distinguish between native and non-native disulfides. We followed the oxidation of RNase A at the defined reaction times by blocking free thiols with AMS, which adds 0.5 kDa for each free sulfydryl group (19). The oxidation state of RNase A can then be monitored using SDS-PAGE. We found that for $A_{CxxC}A'_{AxxA}$, oxidation is compromised and accordingly, it has a marked defect in refolding native RNase A (Fig. 2, *B* and *C*). In contrast, $A_{AxxA}A'_{CxxC}$ and wild-type PDI have a similar ability to catalyze RNase A oxidation (Fig. 2*C*). Therefore, in comparing $A_{AxxA}A'_{CxxC}$ and wild-type PDI, there is discordance between their rate of disulfide formation and the yield of native RNase A (Fig. 2, *B* and *C*).

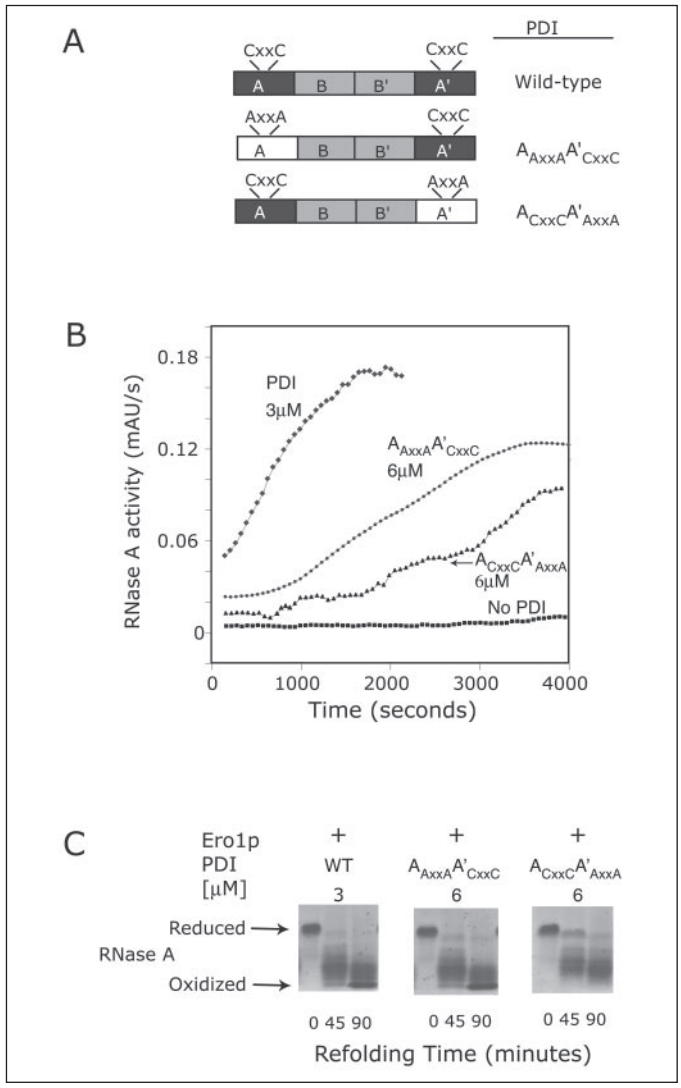

FIGURE 2. **Both of the PDI active site mutants have marked defects in refolding RNase A.** *A*, schematic of the PDI domain architecture and the active site mutants used in this study. A, B, B′, A′ all share the same thioredoxin fold but only A and A′ have the C*x*xC active site. $A_{AxxA}A'_{CxxC}$ refers to the PDI mutant $Cys^{61} \rightarrow Ala,Cys^{64} \rightarrow Ala$ and $A_{CxxC}A'_{AxxA}$ refers to the PDI mutant $Cys^{406} \rightarrow Ala,Cys^{409} \rightarrow Ala$. *B*, both $A_{AxxA}A'_{CxxC}$ and $A_{CxxC}A'_{AxxA}$ have defects in refolding RNase A. The re-oxidation of reduced RNase A (15 μM) was assayed by hydrolysis of cCMP in the presence of Ero1p (0.05 μM), FAD (100 μM), and no PDI (■) or PDI (3 μM) (◆), or $A_{AxxA}A'_{CxxC}$ (6 μM) (●) or $A_{CxxC}A'_{AxxA}$ (6 μM) (▲). *AU* stands for absorbance units. *C*, $A_{AxxA}A'_{CxxC}$ is competent at oxidizing reduced RNase A, while $A_{CxxC}A'_{AxxA}$ is not. Oxidation of reduced RNase A (15 μM) was followed in the presence of Ero1p (0.05 μM), FAD (20 μM), and PDI or $A_{CxxC}A'_{AxxA}$ or $A_{AxxA}A'_{CxxC}$ at the indicated concentrations. Reactions were quenched at the indicated times with AMS. Oxidized and reduced forms of RNase A were distinguished by non-reducing SDS-PAGE.

The PDI Active Sites Have Distinct Roles; the A Domain Is Mainly an Isomerase, while the A′ Domain Is an Efficient Oxidase—To dissect the contributions of the individual active sites to the activity of wild-type PDI, we quantified the initial rates of disulfide bond formation using a dye, ThioGlo1, which reacts rapidly with free thiols to give a fluorescent product (27). We found that at 3 μM, the N-terminal active site ($A_{CxxC}A'_{AxxA}$) has over a 4-fold defect in the initial rate of oxidation when compared with wild-type PDI (Fig. 3*A*). In contrast, as we saw previously $A_{AxxA}A'_{CxxC}$ has a rate of oxidation that is close to that of wild-type PDI, and when the Ero1p concentration is increased modestly (1.3-fold), the initial rates of oxidation can be matched (Fig. 3*A*). However, even under conditions in which the rate of disulfide formation is matched, we found that $A_{AxxA}A'_{CxxC}$ has a much lower yield of functional RNase A than wild-type PDI (Fig. 3*B*).

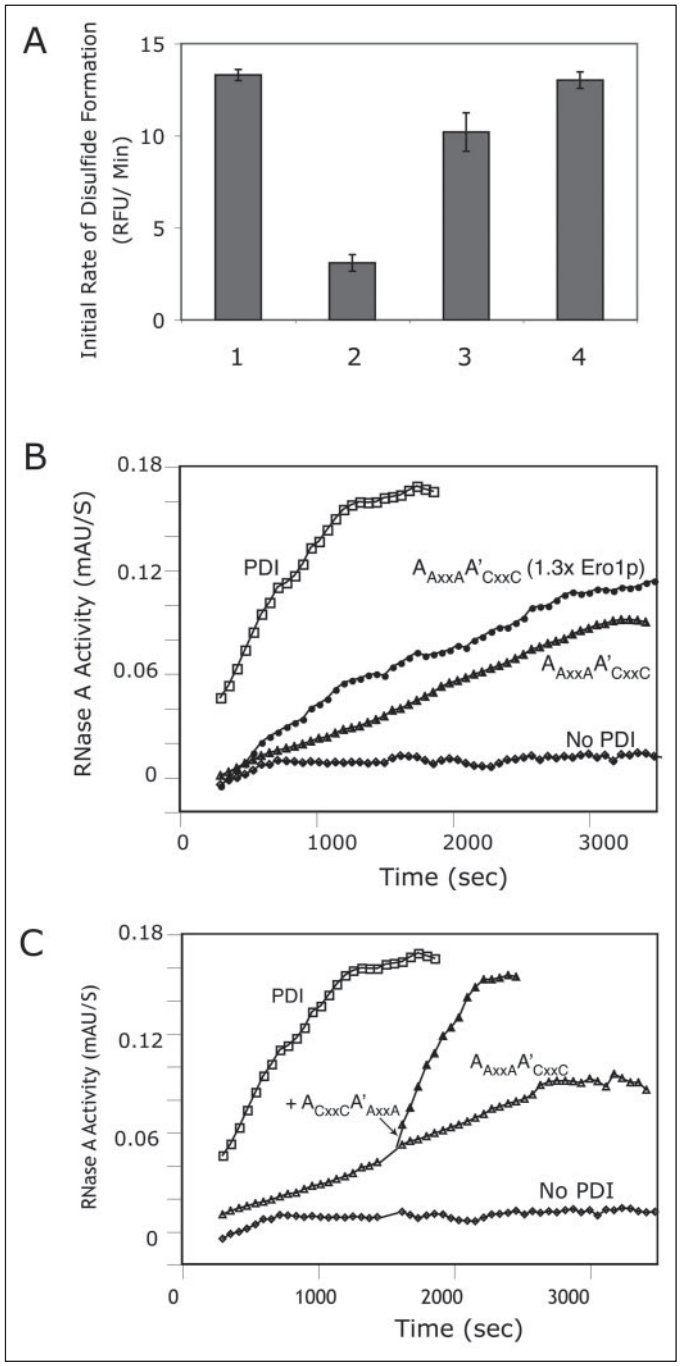

FIGURE 3. **The two active sites of PDI are functionally distinct.** The N-terminal active site functions primarily as an isomerase, while the C-terminal active site is predominantly an oxidase. *A*, the two active sites have unequal contributions to the rate of RNase A oxidation. The rate of re-oxidation of reduced RNase A (15 μM) was followed in the presence of Ero1p (0.05 μM), FAD (20 μM), and PDI (1) or $A_{CxxC}A'_{AxxA}$ (2) or $A_{AxxA}A'_{CxxC}$ (3) at 3 μM or $A_{AxxA}A'_{CxxC}$ at 3 μM with Ero1p (0.065 μM) (4). The rate of oxidation was measured by the loss of free –SH groups over time using the dye ThioGlo1. *RFU* stands for relative fluorescence units. *B*, $A_{AxxA}A'_{CxxC}$ can match the rate of oxidation of wild-type PDI but not the yield of refolded RNase A. The re-oxidation of reduced RNase A (15 μM) was assayed by hydrolysis of cCMP in the presence of Ero1p (0.05 μM), FAD (100 μM), and PDI (3 μM) (□) or $A_{AxxA}A'_{CxxC}$ (3 μM) (▲)or no PDI (♦) or $A_{AxxA}A'_{CxxC}$ (3 μM) and Ero1p (0.065 μM) (●). *C*, $A_{CxxC}A'_{AxxA}$ can complement the isomerization defect of $A_{AxxA}A'_{CxxC}$ in trans. The re-oxidation of reduced RNase A (15 μM) to the native protein was assayed by hydrolysis of cCMP in the presence of Ero1p (0.05 μM), FAD (100 μM), and PDI (3 μM) (□) or no PDI (♦) or $A_{AxxA}A'_{CxxC}$ (3 μM) (△) or $A_{CxxC}A'_{AxxA}$ (3 μM), which is added at 1500 s to the reaction with $A_{AxxA}A'_{CxxC}$ (3 μM) as indicated (▲).

Since rearrangement of incorrect disulfides is a rate-limiting step in refolding RNase A, the failure of $A_{AxxA}A'_{CxxC}$ to refold RNase A may be due to a defect in isomerization (29). Additionally, the presence of the N-terminal active site contributes significantly to the activity of wild-type PDI even though it is a poor oxidase, arguing that the N-terminal active site functions as an isomerase. To determine whether the two active sites are functionally distinct, we tested the possibility that $A_{CxxC}A'_{AxxA}$ can act in trans with $A_{AxxA}A'_{CxxC}$ to overcome its marked defect in refolding RNase A (Fig. 3*C*). Indeed, we found that $A_{CxxC}A'_{AxxA}$ can complement $A_{AxxA}A'_{CxxC}$ in trans even when $A_{CxxC}A'_{AxxA}$ is added well after the oxidation reaction is initiated. The rapid increase in the rate of RNase A refolding seen upon $A_{CxxC}A'_{AxxA}$ addition to pre-oxidized RNase A indicates that it promotes the conversion of non-native disulfides to the native form. Together, $A_{AxxA}A'_{CxxC}$ and $A_{CxxC}A'_{AxxA}$ can match the yield of refolded RNase A by wild-type PDI (Fig. 3*C*).

Thus, we find that there is a functional asymmetry in the PDI active sites with the C-terminal active site acting primarily as an oxidase, while the N-terminal active site acts as an isomerase.

Mechanism of Asymmetry in the PDI Active Sites; the N-terminal Active Site Is Oxidized More Slowly than the C-terminal Active Site—What is the mechanistic basis of the functional asymmetry in the PDI active sites? A previous study has suggested that Ero1p cannot oxidize the N-terminal active site of PDI (30). However, Ero1p was used under conditions in which it was compromised because no additional FAD was present in the reaction, which is known to be required for its activity (19, 22, 25, 30). Additionally, the activity reported was under conditions in which Ero1p was in large excess over PDI (10:1), in contrast to the multiple turnovers that we typically monitor using substoichiometric Ero1p. For these reasons, we systematically explored the ability of Ero1p to oxidize the PDI active sites and analyzed the minimal requirements for recognition by Ero1p.

We used the ThioGlo1 fluorescence assay to establish conditions in which the initial rate of oxidation of PDI is linearly dependent on its concentration and the relative rate of oxidation of the PDI active sites can be compared (Fig. 4*A*). Our data indicate that Ero1p is able to oxidize both PDI active sites and, as seen previously, there is negligible oxidation in the absence of additional FAD (Fig. 4*B*) (19). Since $A_{CxxC}A'_{AxxA}$ appeared to be oxidized more slowly than $A_{AxxA}A'_{CxxC}$, we compared their initial rates of oxidation and found that there is a 2-fold disparity in the ability of Ero1p to oxidize the two active sites (Fig. 4*C*). Additionally, we found that the sum of the initial rates of oxidation of $A_{CxxC}A'_{AxxA}$ and $A_{AxxA}A'_{CxxC}$ is equal to the rate of oxidation of PDI strongly suggesting that the two domains are oxidized independently (Fig. 4*C*).

We also compared the initial rate of oxidation of a series of PDI domain truncations to identify the minimal requirement for functional interaction with Ero1p. We found that Ero1p is able to oxidize the isolated A′ domain indicating that a single thioredoxin domain is sufficient for recognition. However, the rate of oxidation of the A′ domain is half that of $A_{AxxA}A'_{CxxC}$ indicating that the enhanced rate of oxidation of the A′ domain in the full-length protein is contingent on the presence of the A domain (Fig. 4*C*). Additionally, the rates of oxidation of BB′A′, B′A′ and A′ are the same, demonstrating that the loss of either the B or B′ domain does not affect the interaction with Ero1p (Fig. 4*C*). Also, there is no difference in the rate of oxidation of the N- and C-terminal active sites in the PDI truncation mutants (A *versus* A′). This is in contrast to the 2-fold disparity between the rate of oxidation of the N- and C-terminal active sites in the PDI mutants, $A_{CxxC}A'_{AxxA}$ and $A_{AxxA}A'_{CxxC}$.

Thus, we show that the fundamental unit of recognition is the thioredoxin domain as Ero1p can oxidize the isolated A and A′ domains. Our results additionally highlight the role of the thioredoxin domain archi-

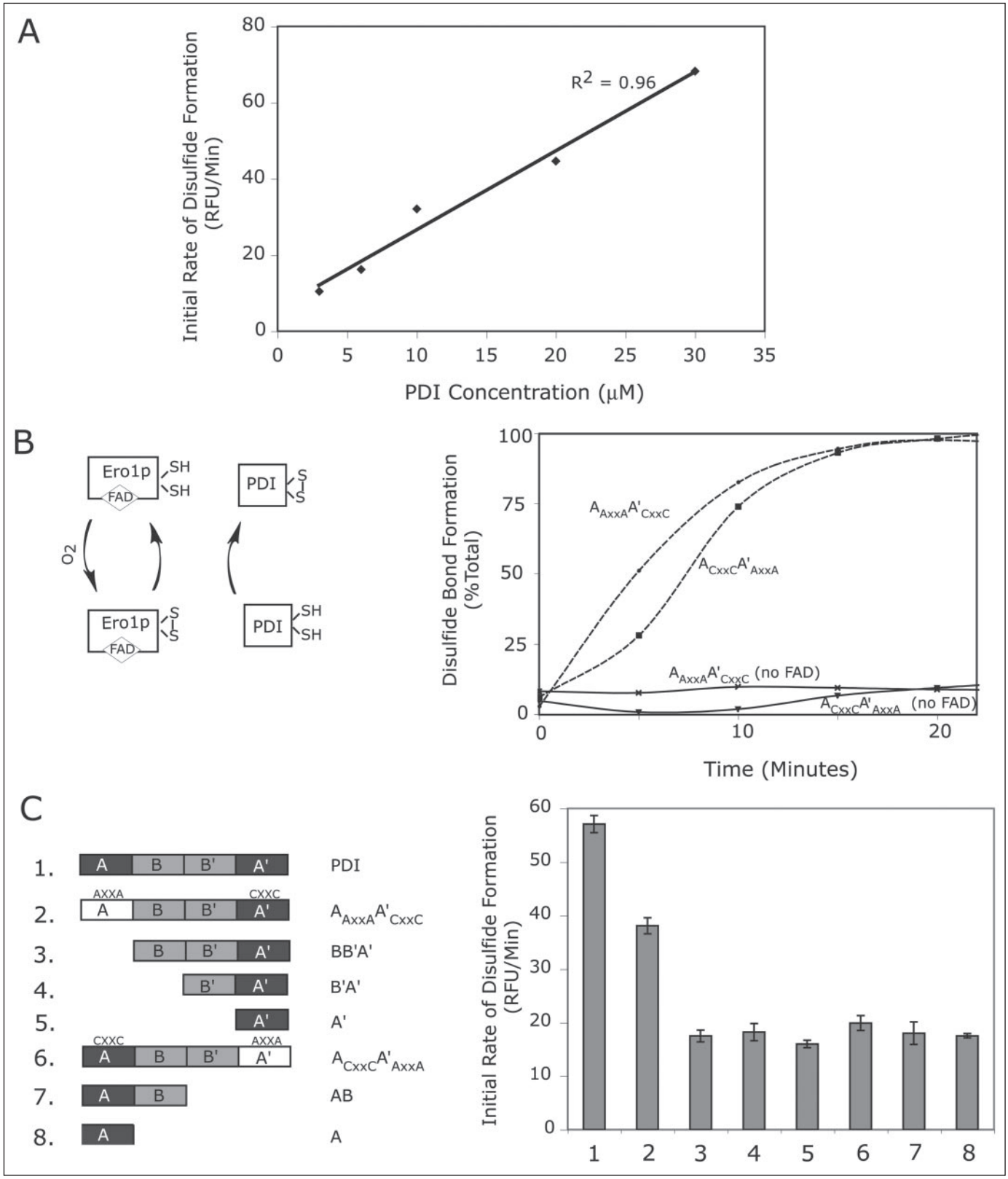

FIGURE 4. **Ero1p can recognize and oxidize both the N- and C-terminal active sites of PDI.** *A*, the rate of oxidation is linearly dependent on PDI concentration. The rate of oxidation of PDI at the indicated concentrations was monitored in the presence of Ero1p (0.3 μM) and/or FAD (20 μM). The initial rate of oxidation was measured by the loss of free –SH groups with time using the dye ThioGlo1. *B*, Ero1p can oxidize both of the PDI mutants $A_{CxxC}A'_{AxxA}$ and $A_{AxxA}A'_{CxxC}$. The rate of oxidation of reduced $A_{CxxC}A'_{AxxA}$ (10 μM) and $A_{AxxA}A'_{CxxC}$ (10 μM) was monitored in the presence of Ero1p (0.3 μM) and/or FAD (20 μM) (schematic on the *left*). The reaction was quenched at the indicated times with ThioGlo1 and the rate of oxidation was monitored by fluorescence. The data are plotted as the percentage of protein that is oxidized over the course of the reaction. *C*, recognition of PDI by Ero1p does not depend on the B or B′ thioredoxin domains. On the *left* is a schematic of the PDI mutants used in these studies. The rate of oxidation of reduced PDI or the indicated PDI mutants (25 μM) was followed in the presence of Ero1p (0.3 μM) and FAD (20 μM). The rate of oxidation was measured by the loss of free –SH groups over time using the dye ThioGlo1.

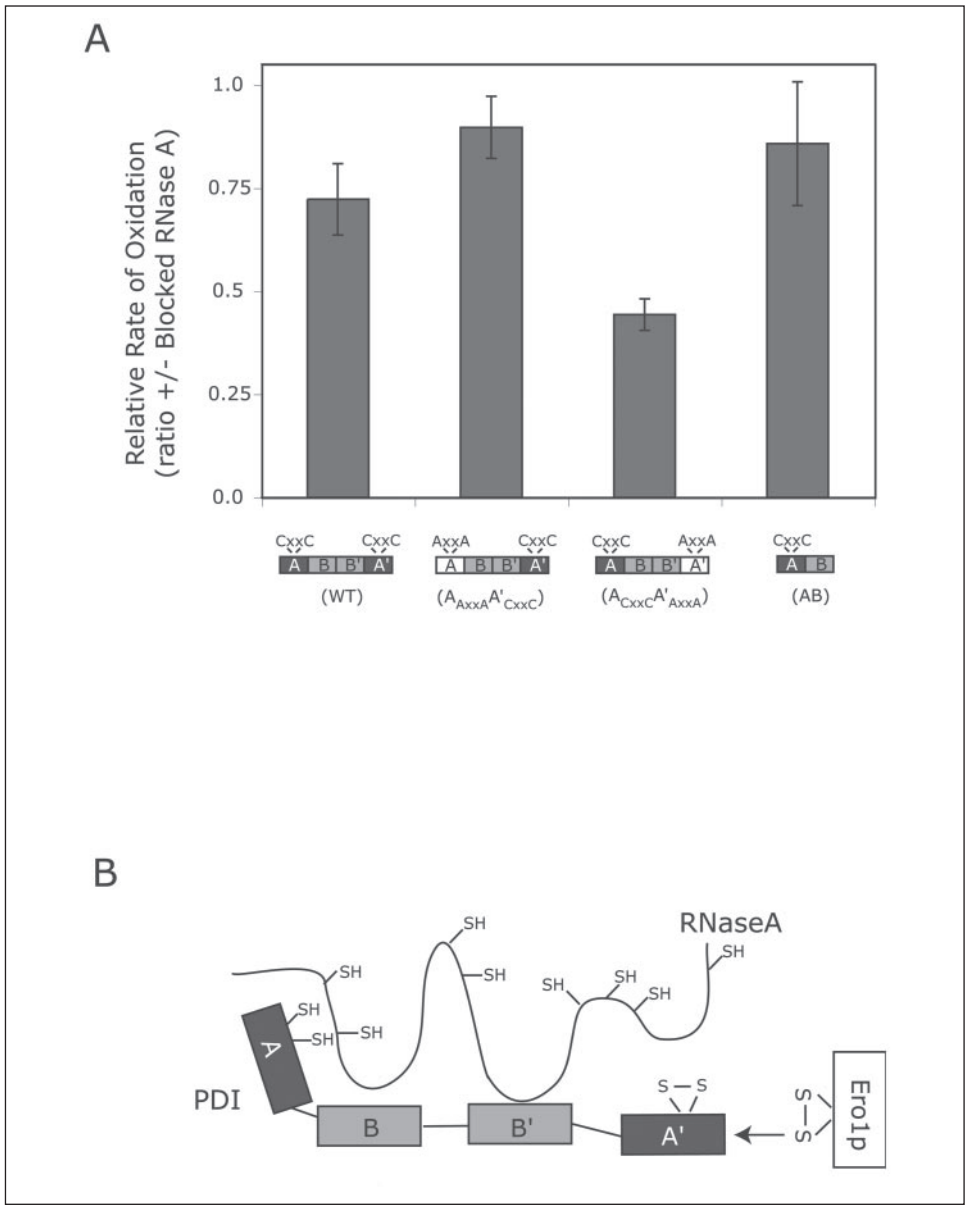

FIGURE 5. **RNase A can modulate the rate of oxidation of the PDI active sites by Ero1p.** *A*, RNase A protects the N-terminal active site of PDI from oxidation and this protection depends on the presence of the substrate binding domains (B′A′). The rate of oxidation of reduced PDI or $A_{CxxC}A'_{AxxA}$ or $A_{AxxA}A'_{CxxC}$ or AB (25 μM) was monitored in the presence of Ero1p (0.3 μM), FAD (20 μM), and/or blocked RNase A (200 μM). The rate of oxidation was measured by the loss of free –SH groups with time using the dye ThioGlo1. The data are presented as ratios of the rates of oxidation in the presence and absence of RNase A. *B*, a schematic model of substrate protection of full-length PDI from Ero1p. The three-dimensional structure of PDI is not known so the interactions between its thioredoxin domains are not defined. The B′ and A′ domains have been shown to be important for the interaction of PDI with RNase A (31) and are also important for the protection of the N-terminal active site from Ero1p (this study).

tecture in establishing asymmetry in the PDI active sites, since the enhanced rate of oxidation of the A′ domain is only apparent in the context of the full-length $A_{AxxA}A'_{CxxC}$.

Substrate Inhibits Ero1-mediated Oxidation of the N-terminal Active Site—Although the intrinsic difference in the rate of oxidation of the PDI active sites by Ero1p may contribute to their functional asymmetry, this 2-fold disparity in the rate of oxidation of the two active sites is not sufficient to fully explain the difference in their ability to oxidize RNase A. For this reason, we explored the possibility that substrate binding to PDI might play a more active role in altering the rate of Ero1p-mediated oxidation. The substrate can affect the rate of the reaction in two ways; first, it is a source of reductant for PDI, and second, it is an unfolded protein that binds to PDI and may alter the ability of Ero1p to interact with PDI. To specifically focus on the effects of substrate binding in the absence of catalysis, we took advantage of the fact that the interaction between PDI and folding substrates can occur in the absence of substrate disulfides (31) and measured the rate of oxidation of PDI in the presence of reduced and alkylated RNase A.

We found that substrate binding specifically protects the first thioredoxin domain from oxidation by Ero1p. The rate of oxidation of $A_{CxxC}A'_{AxxA}$ decreases to 40 ± 4% in the presence of alkylated RNase A (Fig. 5*A*). In contrast, the rate of oxidation of $A_{AxxA}A'_{CxxC}$ is not altered significantly in the presence of this substrate. The effect of substrate binding on the rate of oxidation of wild-type PDI is intermediate (70 ± 9%), which is consistent with its altering the oxidation of the first but not the second active site. Furthermore, we found that the addition of alkylated RNase A does not inhibit the oxidation of the N-terminal active site in the PDI mutant, AB (Fig. 5*A*). Therefore, the B′A′ domains, which have been shown previously to be required for substrate binding, are required for this effect (31).

Thus, the substrate-mediated protection of the N-terminal active site magnifies the intrinsic difference in rate of oxidation of the PDI active sites and further contributes to the asymmetry in their function. It remains to be explored whether the substrate-mediated protection of the A domain results from a direct competition between substrate and Ero1p for PDI recognition (Fig. 5*B*). Alternately, substrate binding could

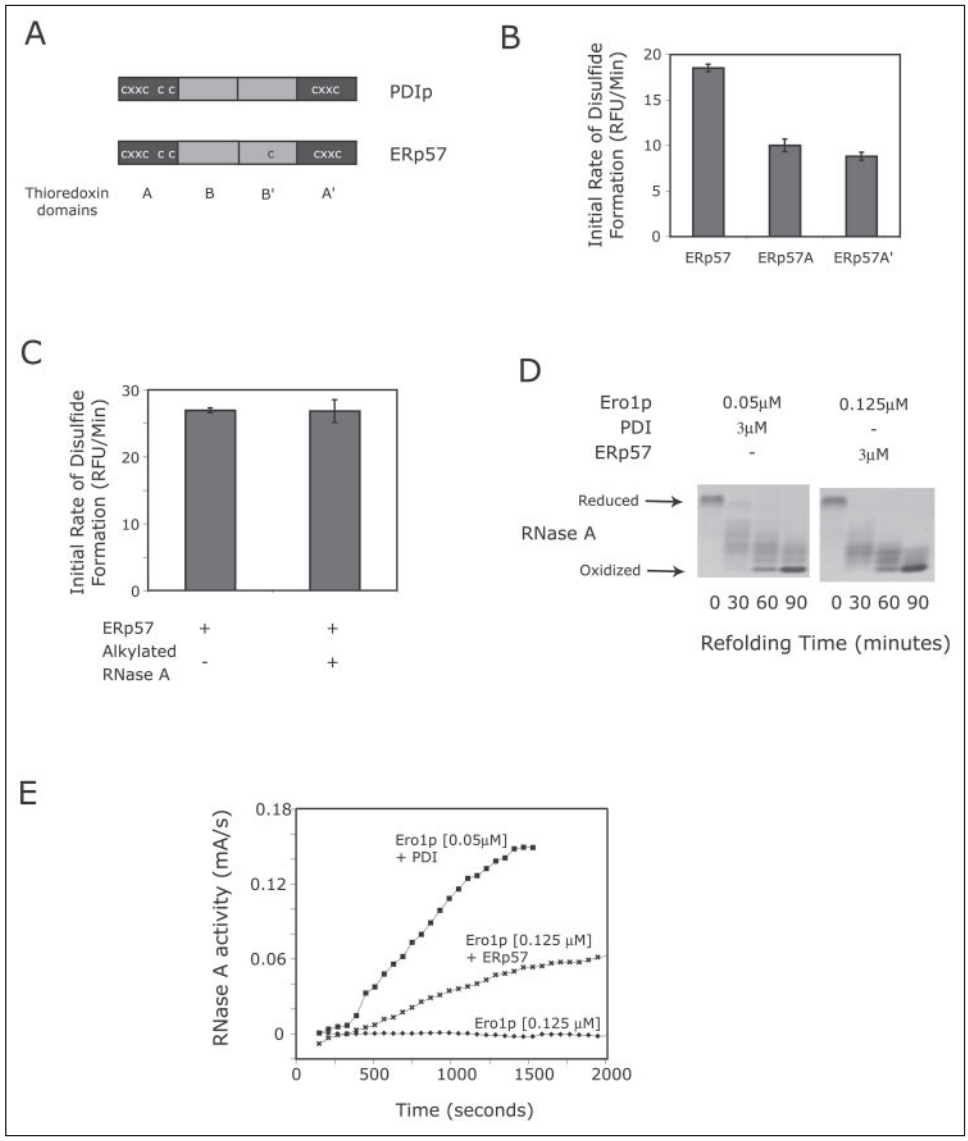

FIGURE 6. **ERp57 is a substrate for oxidation by Ero1p *in vitro* but lacks the asymmetry in its active sites.** *A*, ERp57 is a human PDI related protein that contains two C*xx*C active sites like PDI. It also shares its domain structure with PDI and has four thioredoxin domains A, B, B′, and A′. *B*, ERp57 and the individual ERp57 A and A′ thioredoxin domains are substrates for Ero1p. The rate of oxidation of reduced ERp57 or ERp57 A or A′ domains (25 μM) was followed in the presence of Ero1p (0.3 μM) and FAD (20 μM). The rate of oxidation was measured by the loss of free –SH groups with time using the dye ThioGlo1. *C*, RNase A does not affect the rate of oxidation of ERp57 by Ero1p. The rate of oxidation of reduced ERp57 (25 μM) was monitored in the presence of Ero1p (0.3 μM), FAD (20 μM), and/or blocked RNase A (200 μM). The rate of oxidation was measured by the loss of free –SH groups with time using the dye ThioGlo1. *D*, ERp57 can act as an oxidase *in vitro* and pass oxidizing equivalents from Ero1p to RNase A. Oxidation of reduced RNase A (15 μM) was followed in the presence of Ero1p at the indicated concentrations, FAD (20 μM) and PDI or ERp57. The reactions were quenched at the indicated times with AMS. Oxidized and reduced forms of RNase A were distinguished by non-reducing SDS-PAGE. Note that the amount of Ero1p added was altered so that the overall rate of oxidation of RNase A by ERp57 and by PDI was similar. *E*, given the same rate of oxidation of RNase A, ERp57 is less effective than PDI at isomerization of incorrect disulfides. The re-oxidation of reduced RNase A (15 μM) was assayed by hydrolysis of cCMP in the presence of Ero1p at the indicated concentrations, FAD (100 μM) and PDI (3 μM) (■) or ERp57 (3 μM) (×) or no PDI (♦).

also induce a conformational change in PDI preventing access of Ero1p to the A domain.

Lack of Asymmetry in the PDI Homolog, ERp57, Results in a Defect in Ero1p-mediated Disulfide Folding—To assess whether the features of the interaction between PDI and Ero1p are generally applicable to other disulfide oxidoreductases, we turned to ERp57, a human PDI homolog that shares the ABB′A′ domain architecture (Fig. 6*A*). The specific redox role that ERp57 plays in promoting disulfide-linked folding *in vivo* is not well defined. ERp57 is mostly reduced *in vivo* and its redox state does not change upon overexpression of hEro1α or hEro1β, indicating that it might be a poor substrate for Ero1 *in vivo* (32). Since the substrate-binding (B′) domains in PDI and ERp57 have different specificity, it has been proposed that this might explain why these highly homologous proteins vary in their ability to interact with Ero1 (33, 34). However, this hypothesis is at odds with our result that the B′ domain in PDI is not required for recognition by Ero1p (Fig. 4*C*). For this reason, we assayed the ability of Ero1p to oxidize ERp57 *in vitro*.

We found, using the ThioGlo1 assay, that ERp57 and the individual ERp57 thioredoxin domains, A and A′, are all oxidized by Ero1p *in vitro* (Fig. 6*B*). However, the ERp57 active sites lack intrinsic asymmetry, since the rates of oxidation of the individual thioredoxin domains together add up to the rate of oxidation of ERp57 (Fig. 6*B*). This is in contrast to PDI, where the rates of oxidation of the isolated PDI thioredoxin domains taken out of context of the full-length protein do not add up to the rate of oxidation of wild-type PDI (Fig. 4*C*). We also found that unlike PDI, the presence of alkylated RNase A does not affect the rate of oxidation of ERp57 (Fig. 6*C*).

We, therefore, compared the activity of ERp57 and PDI to specifically investigate whether the asymmetry in the PDI active sites is important for promoting more efficient folding. We matched the rate of RNase A oxidation by ERp57 and Ero1p to the rate with PDI and Ero1p by either altering ERp57 levels (data not shown) or using more Ero1p (Fig. 6*D*). However, when we used the cCMP assay under these conditions, we found that ERp57 has a marked defect in generating native protein (Fig. 6*E*). This discrepancy between the rate of oxidation of RNase A and the yield of native RNase A is similar to the defect observed in the PDI active site mutant $A_{AxxA}A'_{CxxC}$ and illustrates the importance of the asymmetry in the PDI active sites for its role as an effective disulfide isomerase. Our results, however, do not rule out a cellular role for ERp57 as a disulfide isomerase. Since ERp57 functions *in vivo* as part of the calnexin/calreticulin system and specifically interacts with glycosylated substrates, it is possible

that the association between ERp57 and calnexin/calreticulin or its cellular substrates might modify its *in vivo* redox state and enable it to function as a more efficient isomerase.

Taken together, our results show that the domains of PDI have distinct roles in the oxidation and isomerization of substrate disulfides and that the asymmetry in the PDI active sites allows it to more effectively promote native disulfide formation.

DISCUSSION

Our studies provide key insights into how the ER is able to balance the opposing redox processes of disulfide bond oxidation and isomerization that are required to achieve optimal protein folding. We found that there is an asymmetry in the active sites of PDI that enables it to act in a dual capacity of an oxidase and an isomerase. The C-terminal active site is efficient at oxidizing RNase A but is defective at catalyzing the formation of correct disulfides. In contrast, the N-terminal active site is a poor oxidase but it contributes significantly to making functional RNase A because it can isomerize incorrect disulfides. This asymmetry in the PDI active sites is achieved by a compounding of two effects; the substrate-independent enhanced rate of oxidation of the A′ domain and the substrate-mediated inhibition of oxidation of the A domain. We also show that there is no asymmetry in the rate of oxidation of the individual catalytic domains, A and A′. The discrepancy in the rate of oxidation of the two active sites is only observed in the context of the full-length protein and is dependent on the specific order of thioredoxin domains. The resulting disparity in the rate of oxidation of the two active sites ensures that the A′ domain is in the oxidized state, which is required to catalyze disulfide formation, while the A domain is reduced and can promote disulfide isomerization.

Our studies help explain previous studies of the activity of the PDI active site mutants in *S. cerevisiae*. In particular, the C-terminal active site of PDI was shown to be dispensable for proper maturation of procarboxypeptidase Y (pro-CPY), while the loss of the N-terminal active site led to a delay in CPY maturation (43, 44). These *in vivo* results were at odds with the *in vitro* studies of PDI in a glutathione redox buffer in which the C-terminal active site was more efficient at refolding pro-CPY (44). Our data, however, show that in the context of the Ero1p-PDI pathway, the N-terminal active site is an inefficient oxidase but a good isomerase. This suggests that the rate-limiting step in the maturation of CPY, a protein with five disulfides, is disulfide isomerization, which could explain the importance of the N-terminal active site in the folding of CPY *in vivo*. Recent studies in *S. cerevisiae* and in mammalian cell lines have also found that PDI is partially reduced *in vivo* suggesting that the protection of the PDI active sites that we observe *in vitro* may also be observed *in vivo* and implying that there is a pool of reduced PDI active sites that can function as an isomerase *in vivo* (45, 46).

This strategy of disulfide bond formation in the ER represents a markedly different solution from the prokaryotic model. The functional homolog of Ero1p in prokaryotes is DsbB, and both Ero1p and DsbB proteins specifically oxidize a protein with thioredoxin domains (PDI in the case of Ero1p and DsbA in the case of DsbB), which then directly oxidize substrates (2). Bacteria have a separate pathway for catalyzing disulfide bond isomerization, using two key players DsbC and DsbD (35, 36). A functional equivalent of the DsbD pathway has not yet been identified in the ER. In contrast, it now appears that the Ero1p-PDI pathway plays a dual role in both disulfide bond oxidation and isomerization. These different strategies of disulfide bond formation could be a result of the physical differences between the ER and the periplasm. Unlike the periplasm, the ER is bathed in a reducing environment with a flux of glutathione from the cytosol. Previous studies have shown that glutathione provides net reducing equivalents to the ER (18). The presence of a small molecule reductant and the ability of PDI to act as an isomerase could minimize the requirement for a separate disulfide reduction pathway in eukaryotes.

The eukaryotic mechanism has a number of potential advantages over the prokaryotic system. Our experiments show that having a single PDI protein function as a dual oxidase/isomerase is more efficient at folding a model substrate, presumably because of a higher local concentration of both active sites (data not shown). Also, the regulation of PDI redox state by substrates has important implications for oxidative protein folding in the ER. When there are fewer substrates to fold, Ero1p can keep PDI in a completely oxidized state poised for the transfer of disulfides to substrate proteins at a maximal rate. Once PDI engages the substrate, it can then switch to the dual roles of an oxidase and isomerase. In this mode, the ternary complex of Ero1p, PDI, and substrates could permit processivity in disulfide bond formation, magnifying the effect of the asymmetry in the PDI active sites. It is also possible that the nature of the substrate would dictate the extent of protection of the N-terminal active site. This would allow PDI to fine tune the roles of oxidation *versus* isomerization depending on the requirements of the substrate. These advantages of the Ero1p-PDI pathway are particularly well suited for the folding requirements of complex eukaryotic substrates, which in general have larger numbers of disulfides than substrates in bacteria.

Our results also provide insight into the mechanism of recognition of PDI by Ero1p. One suggested model is that the substrate-binding site in PDI, which sequesters unfolded proteins, could also mediate recognition of Ero1p by binding a loop that appears unstructured in the Ero1p crystal structure (22). However, we found that the substrate-binding domain (B′) was not required for productive interaction with Ero1p. We also found that Ero1p is fairly nonspecific in its recognition of thioredoxin domains, which helps explain why the expression of several PDI homologs can rescue the viability of a Δ*pdi1* strain (11, 12, 37). This lack of discrimination seems at odds with the strong preference that Ero1p has for thioredoxin domains over unfolded proteins (19). We speculate that in addition to protein-protein recognition, this specificity might also stem from the highly reactive redox properties of the active site cysteines in thioredoxin domains (reviewed in Ref. 1).

Our studies provide a rationale for why there has been such a proliferation of PDI homologs with combinatorial arrangements of active and inactive thioredoxin domains. Namely, the specific sequence of thioredoxin domains can determine the function of a PDI homolog. We show that the ability of PDI to act as a disulfide bond isomerase requires the presence of both the A domain and the substrate binding domains (B′ and A′). The redox niche occupied by ERp57 and other PDI homologs may be determined by their interactions with different chaperones and substrates. ERp57 has an arrangement of thioredoxin domains similar to PDI, but its substrate binding domain (B′) binds more efficiently to the ER lectins, calnexin, and calreticulin than directly to substrates like RNase A (38). Since ERp57 appeared to be largely reduced *in vivo* (32), it is possible that ERp57 is protected from Ero1 by its interactions with calnexin and calreticulin allowing it to function as a disulfide reductase/isomerase.

More generally, such networks of interactions between the PDI homologs could play a critical role in regulating their function. The PDI homologs, ERp72 and P5, have been suggested to be in complexes with other chaperones in the ER (39). Also, ERdJ5, a mammalian homolog with a DnaJ domain, is thought to be associated with other heat shock proteins (40–42). By working in concert with other proteins, as shown here for PDI and RNase A, PDI homologs might modulate their inter-

actions with Ero1p and thereby ensure a more specialized folding environment tailored to the specific requirements of different substrate proteins.

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