

## The Essential Function of Protein-disulfide Isomerase Is to Unscramble Non-native Disulfide Bonds\*

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**Protein-disulfide isomerase (PDI) is an abundant protein of the endoplasmic reticulum that catalyzes dithiol oxidation and disulfide bond reduction and isomerization using the active site CGHC. Haploid *pdi1Δ* *Saccharomyces cerevisiae* are inviable, but can be complemented with either a wild-type rat PDI gene or a mutant gene coding for CGHS PDI (shufflease). In contrast, *pdi1Δ* yeast cannot be complemented with a gene coding for SGHC PDI. *In vitro*, shufflease is an efficient catalyst for the isomerization of existing disulfide bonds but not for dithiol oxidation or disulfide bond reduction. SGHC PDI catalyzes none of these processes. These results indicate that *in vivo* protein folding pathways contain intermediates with non-native disulfide bonds, and that the essential role of PDI is to unscramble these intermediates.**

Protein-disulfide isomerase (PDI;<sup>1</sup> EC 5.3.4.1) constitutes approximately 2% of the protein in the endoplasmic reticulum (ER). PDI has been shown to catalyze the *in vitro* oxidation of protein sulfhydryl groups and reduction and isomerization of protein disulfide bonds (1, 2; Fig. 1). The products of catalysis by PDI depend on the dithiol/disulfide reduction potential of the substrate and the solution. The enzyme itself can exist in either a reduced or oxidized state or as a mixed disulfide with a substrate. Although PDI is the most efficient known catalyst of oxidative protein folding (3, 4), it also participates in cellular processes that do not exploit its enzymatic activity (5, 6).

Mature PDI from rat contains two active sites with the sequence WCGHCK (7). The C terminus of rat PDI ends with

the sequence KDEL, the signal for retention of proteins in the mammalian ER (8). PDI also has at least one site that can bind to peptides (9, 10). The amino acid sequence of *Saccharomyces cerevisiae* PDI is approximately 30% identical with that of rat PDI, and the regions containing the active sites are conserved completely (11–14). Mature PDI from *S. cerevisiae* contains five putative *N*-glycosylation sites and a C terminus ending with HDEL, the *S. cerevisiae* equivalent of KDEL (13, 15).

The role of PDI *in vivo* is unclear. In *S. cerevisiae*, *pdi1Δ* mutants are inviable (11–14). In *E. coli*, the PDI analog *dsbC* is necessary for the formation of native disulfide bonds in many periplasmic proteins (16). Studies based on the complementation of *pdi1Δ* *S. cerevisiae* have provided some clues as to the role of PDI. Tachibana and Stevens (17) showed that the overexpression of *EUG1*, which codes for an ER protein with active-site sequences WCLHSQ and WCIHSK, allows *pdi1Δ* cells to grow. Also, LaMantia and Lennarz (18) found that *pdi1Δ* cells can be rescued by a mutant PDI that cannot catalyze dithiol oxidation.

To determine why the PDI gene is essential for the growth of *S. cerevisiae*, we have mutated a cDNA that codes for PDI and have tested the ability of the resulting mutant proteins to support the growth of *pdi1Δ* *S. cerevisiae*. These results, coupled with *in vitro* analyses of catalysis, demonstrate that the essential role of PDI is not related to the net formation of protein disulfide bonds. Rather, the role of PDI is to act as a "shufflease," a catalyst of the isomerization of existing disulfide bonds (Fig. 1, bottom).

### EXPERIMENTAL PROCEDURES

**Strains, Media, and Plasmids**—*Escherichia coli* strain CJ236 (*dut ung thi relA*; pCJ105[Cm<sup>r</sup>]) was from Bio-Rad. Diploid yeast strain YPH274 *α/a PDIΔpdi1::HIS3* (homozygous for *ura3-52 lys2-801 ade2-101 trp1-Δ1 his3-Δ200 leu2-Δ1*) was a generous gift from Dr. Tom Stevens. Plasmid pMAL3.1 is a *TRP1* plasmid that directs the expression of rat PDI in *S. cerevisiae* under the control of a hybrid *ADH2-GAPDH* promoter (19). Plasmid pMAL9 is a *TRP1* plasmid that directs the expression of *S. cerevisiae* PDI in *S. cerevisiae* under the control of its own promoter.<sup>2</sup> Other strains, media, and plasmids were obtained as described (19).

Yeast cells were grown at 30 °C in rich or defined medium containing glucose (1% w/v) prepared as described (20). 5-Fluoroorotic acid (5-FOA) addition was as described (21). Visible absorbance measurements were made on a Cary 3 spectrophotometer thermostatted with a Cary temperature controller. Samples were diluted to read A = 0.1–0.5 at 600 nm. Yeast were transformed as described (22).

**DNA Manipulations**—Site-directed mutagenesis was performed on single-stranded DNA isolated from *E. coli* strain CJ236 according to Kunkel and co-workers (23). Other manipulations of DNA, *E. coli*, and *S. cerevisiae* were performed as described (19, 24).

**Plasmid for Production of CGHS PDI and SGHC PDI**—To mutate both active sites in rat PDI, we used a subcloning strategy that took advantage of the *Xma*III site that lies between the regions in pMAL3.1 that code for the two active sites. The *Nco*I/*Xma*III fragment of pMAL3.1 (19) was inserted into an M13mp18-derived plasmid, pMAL10,<sup>2</sup> that had been digested with *Nco*I and *Xma*III, to yield plasmid pMAL11. The codons for cysteine residues in the first active site of the PDI cDNA in pMAL11 were changed to those for a serine residue by using oligonucleotide ML11: GAATTCGGATCGCCCATGG(G/T)CCGGACACTGCAAAGC or ML12: GAATTCGGATCGCCATGGTGTGGGCAT(G/T)CGAAAGCATGG.<sup>3</sup> The regions in the result-

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<sup>1</sup> The abbreviations used are: PDI, protein-disulfide isomerase; ER, endoplasmic reticulum; FPLC, fast protein liquid chromatography; shufflease, C38S/C382S PDI or CGHS PDI; Trx, thioredoxin; DTT, dithiothreitol; 5-FOA, 5-fluoroorotic acid; Mes, 4-morpholineethanesulfonic acid.

<sup>2</sup> M. C. A. Laboissière, S. L. Sturley, and R. T. Raines, submitted for publication.

<sup>3</sup> Oligonucleotides ML11, ML12, ML13, and ML14 were degenerate in that they replaced the cysteine codon with one for either alanine or serine. Only the serine mutants were analyzed in this study.

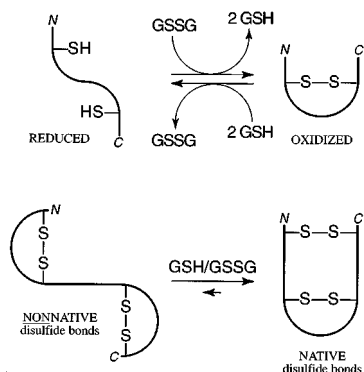


FIG. 1. **Reactions catalyzed by PDI *in vitro*.** PDI catalyzes the oxidation of dithiols and reduction of disulfide bonds (*top*) and the isomerization of disulfide bonds (*bottom*). In this work, the substrate for dithiol oxidation was reduced RNase A, that for disulfide reduction was insulin, and that for disulfide bond isomerization was scrambled RNase A.

ing plasmids that code for PDI were amplified by using the polymerase chain reaction. The amplified fragments were digested with *Nco*I and *Xma*III and ligated to the *Nco*I/*Xma*III fragment of pMAL3.1, generating plasmids pMAL112 (which codes for C35S PDI) and pMAL122 (C38S PDI).

The *Nhe*I/*Xma*III fragment of pMAL3.1 was inserted into pMAL10 that had been digested with *Nhe*I and *Xma*III, to yield plasmid pMAL12. The codons for cysteine residues in the second active site of the PDI cDNA in pMAL12 were changed to those for a serine residue using oligonucleotide ML13: ATGCTCCCTGG(G/T)CCGGACACTG-CAAGCAGCTAGCGCTAGAAAAGCTT or ML14: TCCTGGTGTGGGCA-T(G/T)CGAAGCAGCTAGCGCTAGAAAAGCTT. The regions in the resulting plasmids that code for PDI were amplified by using the polymerase chain reaction. The amplified fragments were digested with *Nhe*I and *Xma*III and ligated to the *Nhe*I/*Xma*III fragment of pMAL112 and pMAL122 generating plasmids pMAL312 (C35S/C379S PDI) and pMAL322 (C38S/C382S PDI).

To discern whether the C-terminal HDEL sequence of *S. cerevisiae* PDI is important for its function *in vivo*, we replaced the C-terminal KDEL sequence of C35S/C379S and C38S/C382S PDI with HDEL. The *Nco*I/*Nhe*I fragments of pMAL312 and pMAL322 were inserted into pMAL5.1 (19) that had been digested with *Nco*I and *Xma*III, to yield plasmids pMAL512 (C35S/C379S/K486H) and pMAL522 (C38S/C382S/K486H).

**Complementation of *pdi1Δ S. cerevisiae***—To determine the role of PDI *in vivo*, we attempted to complement *pdi1Δ S. cerevisiae* by plasmid shuffling using plasmids that code for active-site mutants of rat PDI. Briefly, strain YPH 274 $\alpha$ : $\Delta$ *pdi1::HIS3* was transformed with plasmid pCT37, which contains the yeast PDI gene under the control of the *GAL1* promoter (17). Transformants were selected on solid uracil dropout medium. Sporulation was induced by growth on solid medium containing potassium acetate, and tetrads were dissected onto solid YEP medium containing galactose (2% w/v) and raffinose (1% w/v). Haploid cells complemented by *S. cerevisiae* PDI (*pdi1Δ*/pCT37) were identified by growth on solid medium lacking histidine or uracil. *pdi1Δ*/pCT37 haploids were transformed with plasmids pMAL9, pMAL312, pMAL322, pMAL512, or pMAL522 and plated onto solid tryptophan/uracil dropout medium. Transformants were cultured and then grown on solid medium containing 5-FOA, which selects for cells that have lost the *URA3* plasmid (21). Once growth was observed (which varied from 2 to 5 days), colonies were replica-plated onto solid medium without tryptophan or uracil and onto solid medium containing 5-FOA to repeat the cycle. *trp*<sup>+</sup>/*ura*<sup>-</sup> colonies were isolated from the second round. Cultures of the resulting *his*<sup>+</sup> *trp*<sup>+</sup> *ura*<sup>-</sup> cells (5 for each construct) were grown in YEP(1%)D medium, and doubling times were calculated as described (25).

**Yeast Cell Extracts**—Cultures (50 ml) of *S. cerevisiae* were grown at 30 °C until *A* = 0.5–0.8 at 600 nm. The cells were collected by centrifugation, washed with distilled water (20 ml), and resuspended such that *A* = 20 at 600 nm in 100 mM Tris-H<sub>2</sub>SO<sub>4</sub> buffer, pH 9.4, containing DTT (20 mM). This suspension was incubated for 10 min at 30 °C. Cells were then washed in 1.2 M sorbitol and resuspended such that *A* = 50 at 600 nm in spheroplasting buffer, which was 10 mM potassium phosphate buffer, pH 7.2, containing sorbitol (1.2 M) and DTT (2 mM). Cells were converted into spheroplasts by adding Novozym 234 (Novo Bio-

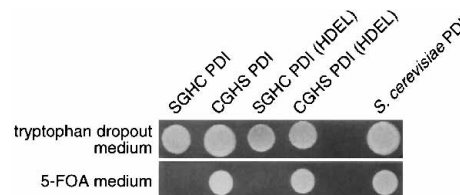


FIG. 2. **Complementation of *pdi1Δ S. cerevisiae*.** Haploid cells carrying a *URA3* plasmid that directs the production of *S. cerevisiae* PDI were transformed with a *TRP1* plasmid that directs the production of a test PDI. Transformants (5 for each plasmid, 1 is shown and the other 4 gave the same result) were cultured, and then grown for 4 days on solid tryptophan dropout medium (*top*) or solid medium containing 5-FOA (*bottom*), which selects for cells that have lost the *URA3* plasmid. *Row 1*, SGHC PDI; *row 2*, CGHS PDI; *row 3*, SGHC PDI (HDEL); *row 4*, CGHS PDI (HDEL); *row 5*, *S. cerevisiae* PDI.

Labs, Bagsvaerd, Denmark) to a final concentration of 3 mg/ml and incubating the resulting solution at 30 °C for 30 min. Spheroplasts were washed twice with 2 volumes of spheroplasting buffer. Spheroplast lysis and protein isolation were then performed as described (26).

**Immunoblots**—Immunoblots were performed as described (19). Antibodies against *S. cerevisiae* PDI were the generous gift of Dr. Tom Stevens.

**Production of Rat PDI in *S. cerevisiae***—PDI was isolated from *S. cerevisiae* culture medium as described (19). Briefly, *S. cerevisiae* strain BJ2168 was transformed with an appropriate plasmid. Transformed cells were grown in filtered YEP(1%)D medium, and the resulting medium was collected and concentrated. PDI was purified by FPLC on a Pharmacia HiLoad 26/60 G-75 gel filtration column, and then on a Pharmacia Mono Q anion exchange column. The fractions containing PDI were >95% pure as assessed by SDS-polyacrylamide gel electrophoresis. The yield of isolated PDI was typically 2 mg/liter culture medium.

**Assays for Enzymatic Activity**—Catalysis of dithiol oxidation was assayed by monitoring the regain in activity of reduced RNase A as follows. Reduced RNase A was prepared by incubating RNase A (5 mg; Sigma) in 1.0 ml of 0.10 M Tris acetate, pH 8.0, containing EDTA (2 mM), guanidinium chloride (6 M), and DTT (0.14 M) for 3 h. The protein was purified by gel filtration chromatography on Bio-Gel P4 resin and then dialyzed exhaustively against 0.10 M acetic acid. Assays were performed at 30 °C in 50 mM Tris-HCl buffer, pH 7.6, containing PDI (0.7–1.4  $\mu$ M), GSH (1 mM), and GSSG (0.2 mM). Reactions were initiated by the addition of reduced RNase A (1.0  $\mu$ l of a 0.50 mg/ml solution in 100 mM acetic acid). At 10-min intervals, aliquots (10  $\mu$ l) were removed and added to 500  $\mu$ l of 100 mM Mes-HCl buffer, pH 6.0, containing poly(C) (10 mM). Ribonuclease activity was monitored by following the change in absorbance at 238 nm.

Catalysis of disulfide reduction was assayed by monitoring the reduction of porcine insulin (Sigma) in the presence of GSH, as described (27).

Catalysis of disulfide isomerization was assayed by monitoring the regain in activity of scrambled RNase A (Sigma), which has non-native disulfide bonds, as described (19).

## RESULTS

**CGHS PDI but Not SGHC PDI Complements *pdi1Δ S. cerevisiae***—Plasmids that direct the expression of mutant forms of rat PDI were made by oligonucleotide-mediated site-directed mutagenesis. Four constructs were tested: mutations in either the first cysteines of both active sites or the second cysteines of both active sites of proteins (designated SGHC PDI and CGHS PDI (or shufflease), respectively) containing the mammalian C-terminal ER retention signal KDEL or the yeast C-terminal retention signal HDEL. These plasmids were transformed into *pdi1Δ S. cerevisiae* to test by plasmid shuffling for suppression of the lethal phenotype. Cells that produce CGHS PDI form colonies, a result similar to that reported previously with a CLHS/CIHS PDI (18). In contrast, cells that produce SGHC PDI were inviable once cured of the *URA3* plasmid (Fig. 2). The identity of the C terminus of PDI (KDEL or HDEL) had no effect on complementation by this assay (Fig. 2).

Immunoblots of membrane fractions extracted before plasmid shuffling showed that rat PDI was present in all transfor-

mants (Fig. 3A). Immunoblots of membrane fractions from cells that complemented the *pdi1Δ* deficiency showed that rat PDI was also present in these cells (Fig. 3B). Immunoblots probed with antibodies to *S. cerevisiae* PDI show that this protein is absent from the rat PDI complemented cells (data not shown).

The abilities of wild-type and mutant PDIs to support the growth of *pdi1Δ S. cerevisiae* is shown in Table I. Cells complemented with PDI containing a C-terminal KDEL or HDEL sequence had indistinguishable doubling times (data not shown). The data described hereafter were obtained from the KDEL constructs.

*Wild-type and CGHS PDI, but Not SGHC PDI, Are Efficient Catalysts of Disulfide Bond Isomerization*—SGHC PDI and CGHS PDI had physical properties similar to those of wild-type PDI and were purified by identical procedures. Wild-type PDI had been shown to be an efficient catalyst of dithiol oxidation (18) and disulfide bond reduction and isomerization (28). In addition, CLHS/CIHS PDI had been shown not to have dithiol oxidation activity (18). Finally, SGHC PDI had been shown not to have disulfide reduction or isomerization activity (28).

Here, we have expanded the assessment of PDI mutants to include all three enzymatic assays on wild-type PDI and the two relevant mutant enzymes. Our results are listed in Table I, and the results of PDI assays are summarized in Table II. Briefly, we found that wild-type rat PDI had dithiol oxidation activity (measured by an increase in activity of reduced RNase A) comparable to that of PDI isolated from bovine liver. SGHC

and CGHS PDI had negligible dithiol oxidation activity. Wild-type rat PDI had disulfide reduction activity (measured by the cleavage of porcine insulin) comparable to that of PDI isolated from bovine liver. SGHC PDI and CGHS PDI had negligible disulfide reduction activity. Wild-type and CHGS rat PDI had isomerization activity (measured by an increase in activity of scrambled RNase A) comparable to PDI from bovine liver. SGHC PDI had negligible isomerization activity.

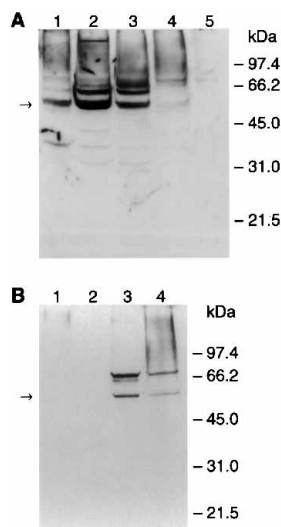
## DISCUSSION

Early work on PDI suggested that its cysteine residues were essential for its enzymatic activity. Carboxymethylation or carbamoylmethylation caused irreversible inactivation (29, 30). In addition, PDI was shown to be inhibited by arsenite or  $Cd^{2+}$ , behavior diagnostic of enzymes with active site dithiol groups (31, 32). Thus, a cysteine residue was suspected to be responsible for the enzymatic activity of PDI.

In 1991, *pdi1Δ S. cerevisiae* cells were shown to be inviable (13). Still, the question remained: What cellular process is impaired by the absence of PDI? PDI catalyzes dithiol oxidation and disulfide bond reduction and isomerization (1). On the other hand, PDI can bind to peptides (9, 10) and is part of cellular complexes in which the role of its enzymatic activity has not been explored (5, 6). These data have led many to suggest that the essential role of PDI is unrelated to its enzymatic activities (18, 33–37).

To illuminate the cellular process that is impaired by the absence of PDI, we have mutated each cysteine residue in its active site and studied the resultant proteins *in vitro* and *in vivo*. Since each PDI monomer has two CGHC active sites, our two mutant proteins are actually double mutants in which either the first or second cysteine residue in each active site is replaced by a serine. We find that CGHS PDI and wild-type PDI are able to complement a *pdi1Δ* strain of *S. cerevisiae* (Table I). In contrast, SGHC PDI is unable to compensate for this deficiency.

Replacing two sulfur atoms with oxygen atoms is unlikely to have a significant impact on the ability of PDI to bind to peptides or to otherwise act in nonenzymic roles. The mutations do, however, have a significant effect on catalysis by PDI. The results of *in vitro* PDI assays show that CGHS PDI catalyzes the shuffling of disulfide bonds with efficiencies comparable to that of the wild-type enzyme (Table I). But, unlike the wild-type enzyme, CGHS PDI does not catalyze the oxidation of dithiols or the reduction of disulfide bonds. Apparently, the ability of PDI to form an intramolecular disulfide bond is necessary for it to catalyze the oxidation or reduction of a substrate efficiently. SGHC PDI catalyzes none of these three processes. Thus, the essential function of PDI is enzymic, but does not relate to the net formation of disulfide protein bonds. Rather,



**FIG. 3. Membrane-bound protein in complemented *pdi1Δ S. cerevisiae*.** Immunoblots were probed with antibodies to bovine PDI. *A*, before plasmid shuffling. Lane 1, SGHC PDI; lane 2, CGHS PDI; lane 3, SGHC PDI (HDEL); lane 4, CGHS PDI (HDEL); lane 5, *S. cerevisiae* PDI. *B*, after plasmid shuffling. Lane 1, *S. cerevisiae* PDI; lane 2, empty; lane 3, CGHS PDI; lane 4, CGHS PDI (HDEL).

TABLE I  
Properties of wild-type and mutant PDIs

PDI	Doubling time of complemented <i>pdi1Δ S. cerevisiae</i> <sup>a</sup>	Dithiol oxidation activity <sup>b</sup>	Disulfide reduction activity <sup>c</sup>	Disulfide isomerization activity <sup>d</sup>
			units/mg	
Wild-type (bovine)	ND	8.5 ± 0.8	0.41 ± 0.04	0.65 ± 0.01
Wild-type (rat)	1.8 ± 0.2	7.0 ± 0.7	0.33 ± 0.03	0.55 ± 0.05
CGHS (rat)	2.3 ± 0.6	0.18 ± 0.02	0.02 ± 0.01	0.51 ± 0.16
SGHC (rat)	NC	0.01 ± 0.01	0.01 ± 0.01	0.02 ± 0.02

<sup>a</sup> Relative to cells complemented with *S. cerevisiae* PDI. ND, not determined; NC, no complementation.

<sup>b</sup> One unit will catalyze the reactivation of 1 nmol of reduced RNase A per min at pH 7.6 in the presence of GSH (1.0 mM) and GSSG (0.20 mM).

<sup>c</sup> One unit will catalyze the reduction of the disulfide bonds in 1 μmol of insulin per min at pH 7.2 in the presence of GSH (0.50 mM).

<sup>d</sup> One unit will catalyze the reactivation of 1 nmol of scrambled RNase A per min at pH 7.6 in the presence of GSH (1.0 mM) and GSSG (0.20 mM).

TABLE II  
Summary of reactions catalyzed by wild-type and mutant PDIs

PDI	Dithiol oxidation	Disulfide reduction	Disulfide isomerization
Wild-type	Yes (18)	Yes (28)	Yes (28)
CGHS	No (this work) <sup>a</sup>	No (this work)	Yes (this work)
SGHC	No (this work)	No (28)	No (28)

<sup>a</sup> The same result was obtained when the two active sites in PDI were changed to CLHS and CIHS, as in Eug1p (18).

the role of PDI *in vivo* is to act as a shufflease (Fig. 1, bottom).

Our results and those of others expose the critical functional group in the CXXC motif of PDI. The *S. cerevisiae* EUG1 gene complements *pdi1Δ S. cerevisiae*. Each active site of Eug1p (WCLHSQ and WCIHSK) contains only a single cysteine residue (17). Wild-type Eug1p is therefore analogous to the shufflease mutant of PDI. Thioredoxin (Trx) catalyzes disulfide bond reduction in the cytosol of eukaryotes and prokaryotes in the active site: WCGPCK (38). Although the three-dimensional structure of PDI is unknown, that of *E. coli* Trx has been determined by both x-ray diffraction analysis and NMR spectroscopy (39, 40). In the Trx structure, the most pronounced deviation from an almost spherical surface is a protrusion formed by residues 29–37, which includes the active site. The sulfhydryl group of Cys-32, which has a low  $pK_a$ , is exposed to the solvent while that of Cys-35 is recessed. The results of chemical modification studies and  $pK_a$  determinations on PDI are parallel to those on Trx (41), suggesting that the reactivity of the active sites is similar. In addition, PDI is a substrate for thioredoxin reductase, which suggests that the three-dimensional structures of the active sites are similar. Recently, we demonstrated that CGPS Trx but not SGPC Trx can complement *pdi1Δ S. cerevisiae*.<sup>4</sup> Thus, the essential functional group in the CXXC motif is the sulfhydryl group of the N-terminal cysteine residue.

If a CXXS sequence can replace the CXXC motif, why does PDI have a CXXC motif? A CXXC motif could be less susceptible to inactivation by adventitious oxidation to a hindered mixed disulfide or a sulfenic acid (S-OH) because it can escape by forming an intramolecular disulfide bond. In addition, having CXXC and CXXS motifs from endogenous PDI and Eug1p, respectively, could provide cells with a selective advantage.

Catalysis of dithiol oxidation or disulfide bond reduction depends on the redox environment (42, 43). In contrast, during catalysis of disulfide bond isomerization, the substrate does not undergo a net change in oxidation state (Fig. 1). The simplest mechanism for catalysis of an isomerization reaction begins with the attack of a thiolate ion on a protein disulfide, forming a mixed disulfide (44). Then, the protein thiolate produced can attack another protein disulfide bond. Finally, the resulting thiolate can attack the mixed disulfide to release the catalyst, unaltered. Such an isomerization reaction would be driven by the search for the most stable conformation of the substrate protein.

Much evidence suggests that dithiol oxidation is random during the early stages of protein folding (45, 46). Classic studies on the oxidative folding of reduced bovine protease trypsin inhibitor suggest that non-native intermediates accumulate during the folding process (47). In contrast, recent work argues that the well-populated intermediates contain only native disulfide bonds (48). Still, to reach the final conformation, these intermediates must rearrange by forming species with non-native disulfide bonds. PDI has been shown to catalyze this process by rescuing kinetically trapped intermediates (49).

Thus, PDI activity may be required either in a normal protein folding pathway or for rescuing proteins that have become misfolded or aggregated. Our results link the disulfide bond isomerization activity of PDI with cell viability. Thus, as proposed by Anfinsen (50) more than 30 years ago, the essential function of PDI is to isomerize non-native disulfide bonds, to be a shufflease.

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<sup>4</sup> P. T. Chivers, M. C. A. Laboissière, and R. T. Raines, submitted for publication.