

## Forum Review

# Catalysis of Protein Folding by Protein Disulfide Isomerase and Small-Molecule Mimics

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### ABSTRACT

Protein disulfide isomerase (PDI) catalyzes the formation of native disulfide pairings in secretory proteins. The ability of PDI to act as a disulfide isomerase makes it an essential enzyme in eukaryotes. PDI also fulfills other important roles. Recent studies have emphasized the importance of PDI as an oxidant in the endoplasmic reticulum. Intriguing questions remain regarding how PDI is able to catalyze both isomerization and oxidation *in vivo*. Studies of PDI and its homologues have led to the development of small-molecule folding catalysts that are able to accelerate disulfide isomerization *in vitro* and *in vivo*. PDI will continue to provide both an inspiration for the design of such artificial foldases and a benchmark with which to gauge the success of those designs. Here, we review current understanding of the chemistry and biology of PDI, its homologues, and small molecules that mimic its catalytic activity. *Antioxid. Redox Signal.* 5, 413–424.

### INTRODUCTION

**P**ROTEIN DISULFIDE ISOMERASE (PDI; EC 5.3.4.1) was first identified 40 years ago as an enzyme that catalyzes the activation of reduced, and thus inactive, ribonuclease A (RNase A) (29). Anfinsen and his co-workers went on to hypothesize that the primary role of PDI is to be a “general and nonspecific catalyst for disulfide interchange in proteins containing disulfide bonds” (28). From their experiments with reduced RNase A (rRNase A), they suggested that the formation of native disulfide bonds in proteins begins with the uncatalyzed air oxidation of dithiols, followed by the PDI-catalyzed isomerization of non-native disulfide bonds. More recent studies have revealed that an ensemble of enzymes and chaperones are involved in the pathway of native disulfide formation (94, 101). Still, PDI remains at the center of this complex biological process.

### PROPERTIES OF PDI

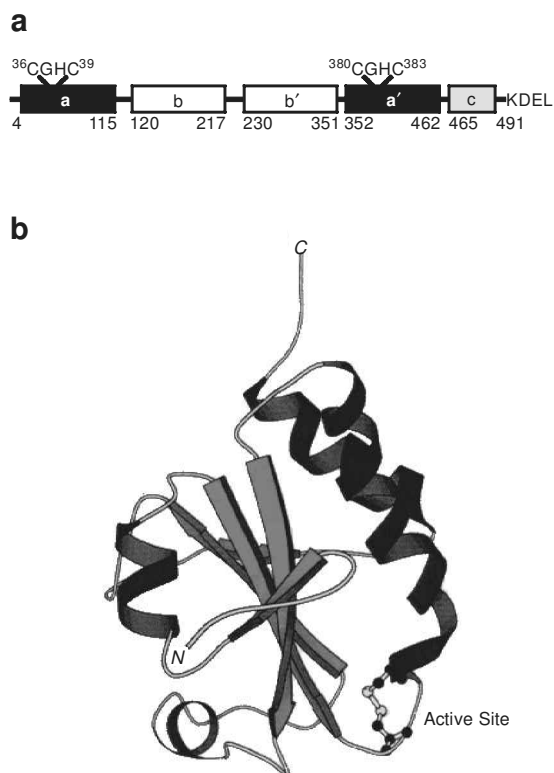
PDI is a 57-kDa protein that resides in the endoplasmic reticulum (ER) of eukaryotic cells. There, PDI catalyzes the

formation, reduction, and isomerization of disulfide bonds in newly synthesized proteins (25). Independent of its catalytic activity, PDI exhibits chaperone activity by inhibiting the aggregation of unfolded proteins (6, 71) and is a member of at least two multimeric enzyme complexes: prollyl 4-hydroxylase (45, 69) and microsomal triglyceride transfer protein (51, 89).

PDI has five distinct structural domains (a, a', b, b', and c; Fig. 1a) as deduced from its primary and tertiary structure (15). The catalytic a and a' domains are homologous, and each contains a Cys–Gly–His–Cys (CGHC) active-site sequence (Fig. 1b). The b and b' domains are also homologous to the a and a' domains (15, 42). The role of the b' domain is to bind substrate proteins (7, 44). The cationic c domain is not required for enzymatic activity (46) and ends with a C-terminal ER retention signal (KDEL in rat and human; HDEL in yeast) (60). The three-dimensional structure of intact PDI is not known, but structures of the individual a, a', b, and b' domains reveal that each has a fold characteristic of thioredoxin (Trx; Fig. 1b) (41, 42).

PDI is a member of the Trx family of proteins, which is characterized by the ability to catalyze thiol–disulfide interchange reactions. Proteins of this family all share a common

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**FIG. 1. PDI is composed of four Trx-like domains. (a).** The a and a' domains each contain a CGHC active site. The b and b' domains are likely involved in substrate binding. The c domain is cationic and ends with a C-terminal ER-retention sequence. The numbering system used here is based on the sequence of rat PDI (15). **(b).** The Trx fold of the oxidized a domain of PDI as revealed by NMR spectroscopy (41). The CGHC active site is at the N-terminus of an  $\alpha$  helix.

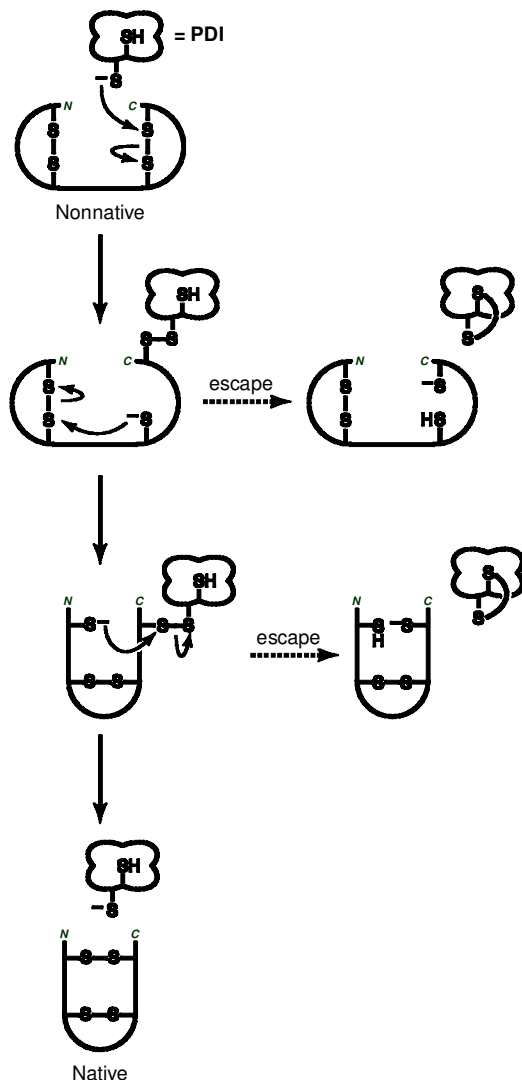
Cys–Xaa–Xaa–Cys (CXXC, where X refers to any amino acid) motif in their active site(s). The N-terminal cysteine residue in this motif has high reactivity at physiological pH, due in part to its location on the surface of the protein (Fig. 1b) and its depressed  $pK_a$  value (10). The family contains proteins with redox properties that range from strongly reducing [Trx,  $E^{\circ'} = -0.270$  V (48)] to strongly oxidizing (DsbA,  $E^{\circ'} = -0.120$  V (35, 36)). The proteins are therefore equipped to perform specialized roles in a variety of cellular compartments.

## MECHANISM OF DISULFIDE ISOMERIZATION

A simple mechanism for disulfide isomerization requires only one reactive thiolate in the active site of PDI. In this scenario (Fig. 2), the more reactive N-terminal cysteine residue of its CXXC motif [ $pK_a = 6.7$  (34)] provides the active-site thiolate. Nucleophilic attack of the thiolate on a nonnative disulfide bond results in the formation of a mixed-disulfide intermediate between enzyme and substrate (11, 13). Conformational changes in the substrate then allow a substrate thiolate to initiate disulfide rearrangements that lead, ultimately,

to the formation of native disulfide bonds and the release of PDI. Based on this mechanism, a CXXS motif should be as efficient as a CXXC motif in catalysis of disulfide isomerization. Yet experimental evidence indicates otherwise.

Eug1p, a homologue of PDI, is a nonessential luminal protein in *Saccharomyces cerevisiae* that contains the active-site sequences CLHS and CIHS (80). The *in vitro* isomerase activity of wild-type Eug1p is low using both reduced procarboxypeptidase Y (proCPY) and scrambled proCPY (which contains nonnative disulfide bonds) as substrates (62). In contrast, a variant of Eug1p in which the CXXS motifs are re-



**FIG. 2. Simple mechanism of disulfide isomerization (11).** Isomerization begins with the nucleophilic attack of a thiolate provided by the catalyst (such as PDI) to form a mixed disulfide intermediate between catalyst and substrate. Additional intramolecular thiol–disulfide interchange reactions are performed by substrate thiols. If these interchange reactions are slow, then a second thiol in the catalyst can provide an escape route, releasing trapped intermediates by reduction of the mixed disulfide bond (85). Native disulfide bonds can then be formed by reoxidation.

placed with CXXC motifs is an efficient catalyst of the folding of scrambled proCPY (62). In addition, CXXC Eug1p catalyzes the folding of scrambled proCPY and reduced proCPY folding at an almost identical rate (62). These experiments support the earlier proposal that isomerization limits catalysis of oxidative folding by enzymes containing a CXXC motif (28), and indicate that the second cysteine residue enhances catalysis.

The second active-site cysteine residue is likely to be important in rescuing trapped intermediates that can accumulate during catalysis (Fig. 2) (85). Complex protein substrates can be slow to rearrange and become trapped in nonnative configurations that prevent successful folding. The C-terminal CXXC cysteine residue provides a mechanism for escape from this obstacle to efficient folding. The presence of two cysteine residues permits PDI to reduce trapped nonnative disulfide bonds and then reoxidize the substrate to form, ultimately, the native disulfide bonds. Recent results indeed indicate that the isomerization of scrambled RNase A (sRNase A; a complex substrate with eight randomly oxidized cysteine residues) by PDI involves cycles of reduction and reoxidation (77).

### REGULATING $E^{\circ'}$ AND $pK_a$

The ability of an oxidoreductase to be an efficient isomerase is governed by the reduction potential of its active-site disulfide bond ( $E^{\circ'}$ ) and the acid dissociation constant of its nucleophilic active-site thiol ( $K_a$ ) (8). For efficient substrate turnover and regeneration of catalyst, an isomerase must strike a balance between the dithiol and disulfide forms. An  $E^{\circ'}$  value that is too low (that is, too negative) will destabilize the dithiol form and provide too little reactive thiolate to initiate attack of a nonnative disulfide bond. The CGPC active site of Trx, for example, has  $E^{\circ'} = -0.27$  V (48) and is an effective reducing agent. An enzyme that contains an active site with an  $E^{\circ'}$  value that is too high will not be able to release trapped intermediates. The CPHC active site of DsbA has  $E^{\circ'} = -0.12$  V and is an effective oxidizing agent, but is inefficient at disulfide isomerization (Table 1) (35, 36, 99).

The thiol  $pK_a$ , like the disulfide  $E^{\circ'}$ , must be in balance. An active-site cysteine residue not only acts as a nucleophilic thiolate to initiate catalysis, but also acts as a leaving group to

allow for thiol–disulfide interchange (27). Hence, an isomerase will be optimal if its thiol  $pK_a$  equals the pH of the environment, which is near 7.0 in the ER (37). Although the  $pK_a$  of a typical cysteine residue is 8.7 (79), a thiol  $pK_a$  that is closer to 7.0 is more optimal for an oxidoreductase in the ER.

The N-terminal cysteine residue of the CXXC motif in Trx-like enzymes consistently has a depressed  $pK_a$  value [6.7 for PDI (34)]. This  $pK_a$  depression is consistent with the location of this residue at the N-terminus of an  $\alpha$ -helix (Fig. 1b) (21). The negative charge on the thiolate of the N-terminal cysteine residue is stabilized by interactions with the positive end of the  $\alpha$ -helix dipole (47). Changes in the intervening –XX– residues can enhance or diminish this Coulombic interaction and thereby decrease or increase, respectively, the  $pK_a$  of the N-terminal cysteine residue (Table 1) (8).

Variations in the –XX– residues of Trx-like enzymes can also modulate the reduction potential of its active sites (Table 2). The variants can differ in thiol–disulfide interchange activity. Altering the residues in Trx can produce a more efficient oxidant (8, 32, 39, 48), whereas changing the residues in DsbA can make it a more potent reductant (32). The values of  $pK_a$  and  $E^{\circ'}$  are related, as a more acidic thiol (lower  $pK_a$ ) produces a less stable disulfide bond (higher  $E^{\circ'}$ ) (9). For –XX– variants of DsbA, changes in the disulfide  $E^{\circ'}$  correlate well with changes in the  $pK_a$  of the N-terminal cysteine residue of the active site (32). On the other hand, active-site variants of Trx with different intervening residues reveal that thiol  $pK_a$  does not exclusively determine disulfide  $E^{\circ'}$  (Table 2) (8, 10). Three active-site variants of Trx (CGHC Trx, CVWC Trx, and CWGC Trx) are able to catalyze the isomerization of disulfide bonds *in vivo* (8). For these variants, the changes in  $E^{\circ'}$  do not correlate with changes in  $pK_a$  (Table 2), which is consistent with the presence of a  $pK_a$ -independent term in the relevant form of the Nernst equation (9, 10):

$$E = E^{\circ} - \frac{RT}{nF} \ln \left( \frac{\alpha_0 F_{P(SH)_2}}{[H^+]^2 F_{PS_2}} \right) \quad (1)$$

where  $E^{\circ}$  is independent of  $pK_a$  and refers to the standard reduction potential,  $\alpha_0$  refers to the fraction of fully protonated dithiol species, and  $F_{P(SH)_2}$  and  $F_{PS_2}$  are the formal (*i.e.*, total) concentrations for the reduced and oxidized molecules, respectively.

TABLE 1. PROPERTIES OF OXIDOREDUCTASES

Protein	Active-site sequence	$E^{\circ'}$ of CXXC (V)	$pK_a$ of CXXC	Primary role in the cell
Rat PDI	WCGHCK	−0.180 <sup>a</sup>	6.7 <sup>b</sup>	Oxidase/isomerase
<i>E. coli</i> Trx	WCGPCK	−0.270 <sup>c</sup>	7.5 <sup>d</sup>	Reductase
<i>E. coli</i> DsbA	FCPHCY	−0.120 <sup>e</sup>	3.3 <sup>f</sup>	Oxidase

<sup>a</sup>Determined from the equilibrium constant with GSH/GSSG and Trx (53).

<sup>b</sup>Determined from the rate of inactivation by alkylation (34).

<sup>c</sup>Determined from the equilibrium constant for the thioredoxin reductase-catalyzed reaction with NADPH/NADP<sup>+</sup> (48).

<sup>d</sup>Determined by <sup>13</sup>C-NMR spectroscopy for the state in which Asp26 and Cys35 are protonated (10).

<sup>e</sup>Determined from the equilibrium constant with GSH/GSSG (35, 36).

<sup>f</sup>Determined by ultraviolet spectroscopy (36).

TABLE 2. PROPERTIES OF ACTIVE-SITE VARIANTS OF TRX

<i>Protein</i>	<i>pK<sub>a</sub> of CXXC</i>	<i>E°' of CXXC (V)</i>	<i>Relative doubling time of complemented pdi1 Δ yeast</i>
Yeast PDI	ND	ND	1.0
Rat PDI	6.7 <sup>a</sup>	−0.180 <sup>b</sup>	1.8 ± 0.2 <sup>c</sup>
CGPC Trx	7.5 <sup>d</sup>	−0.270 <sup>e</sup>	NC
CGPS Trx	ND	—	4.3 ± 0.5 <sup>f</sup>
CGHC Trx	ND	−0.235 <sup>e</sup>	4.4 ± 0.8 <sup>f</sup>
CVWC Trx	6.2 <sup>d</sup>	−0.230 <sup>g</sup>	3.8 ± 0.4 <sup>f</sup>
CWGC Trx	6.1 <sup>d</sup>	−0.200 <sup>g</sup>	2.2 ± 0.2 <sup>f</sup>

ND, not determined; NC, no complementation.  
<sup>a</sup>Determined from the rate of inactivation by alkylation (34).  
<sup>b</sup>Determined from the equilibrium constant with GSH/GSSG and Trx (53).  
<sup>c</sup>Data from ref (49).  
<sup>d</sup>Determined by <sup>13</sup>C-NMR spectroscopy for the state in which Asp26 and Cys35 are protonated (10).  
<sup>e</sup>Determined from the equilibrium constant of the thioredoxin reductase-catalyzed reaction with NADPH/NADP<sup>+</sup> (48).  
<sup>f</sup>Data from ref (8).  
<sup>g</sup>Determined from the equilibrium constant of the thioredoxin reductase-catalyzed reaction with NADPH/NADP<sup>+</sup> (8).

The effective concentration of two thiols reports on their tendency to form an intramolecular disulfide bond. In Trx-like proteins, the effective concentration of thiols in the active site is determined largely by the three-dimensional structure of the protein (3, 9). The insertion of a tryptophan residue in CVWC Trx and CWGC Trx could destabilize the active-site disulfide bond simply by providing steric hindrance to its formation. Alternatively, removal of the proline residue in the CVWC and CGHC variants could increase the reduction potential by increasing the conformational entropy of the polypeptide chain, and thereby stabilizing the reduced form relative to the oxidized form. From such changes, a set of homologous oxidoreductases has evolved to carry out a variety of redox functions in different cellular environments (Table 1).

DISULFIDE ISOMERIZATION—THE ESSENTIAL FUNCTION

PDI is required for the viability of *S. cerevisiae* (20, 50, 75, 81). Of all the cellular roles of PDI, its most important

function is the isomerization of nonnative disulfide bonds (11, 49). Replacing PDI with a variant in which both active-site sequences are CGHS, instead of CGHC, restores viability to *pdi1Δ S. cerevisiae* (Table 3). Although this PDI variant has low dithiol oxidation activity and low disulfide reduction activity, it is proficient in its catalysis of disulfide isomerization (Table 3) (49, 86). A PDI variant that contains SGHC active-site sequences is unable to catalyze the formation, reduction, or isomerization of disulfide bonds, and does not complement a *pdi1* deletion (Table 3).

Further support for the conclusion that isomerization is the essential function of PDI comes from *in vivo* studies with PDI homologues. Overproduction of Eug1p (with its CLHS and CIHS active sites) is able to rescue *pdi1Δ S. cerevisiae*, even though the isomerization activity of Eug1p is less than that of PDI (80). Trx is unable to restore viability to *pdi1Δ* yeast because of its low *E°'* value (Table 2) (8). A Trx variant in which the active site is replaced with CGPS is, however, able to complement a *pdi1* deletion (Table 2) (8). In Eug1p and CGPS Trx, catalysis of dithiol oxidation and disulfide reduction is not efficient because neither enzyme can form a disulfide bond in its active site. Nonethe-

TABLE 3. PROPERTIES OF ACTIVE-SITE VARIANTS OF PDI

<i>PDI<sup>a</sup></i>	<i>Dithiol oxidation activity<sup>b</sup></i>	<i>Disulfide reduction activity<sup>c</sup></i>	<i>Disulfide isomerization activity<sup>d</sup></i>	<i>Doubling time of complemented pdi1 Δ S. cerevisiae<sup>e</sup></i>
CGHC	100	100	100	1.8 ± 0.2
CGHS	3	6	93	2.3 ± 0.6
SGHC	0	3	4	NC

Data were obtained from ref. (49). NC, no complementation.  
<sup>a</sup>For each protein, the sequence indicated is present in both active sites of rat PDI.  
<sup>b</sup>Percentage of wild-type PDI activity for the activation of rRNase A.  
<sup>c</sup>Percentage of wild-type PDI activity for the reduction of the disulfide bonds in insulin.  
<sup>d</sup>Percentage of wild-type PDI activity for the activation of sRNase A.  
<sup>e</sup>Relative to cells complemented with *S. cerevisiae* PDI.

less, both enzymes are able to catalyze disulfide isomerization and thereby endow a cell with the activity that is necessary for its survival.

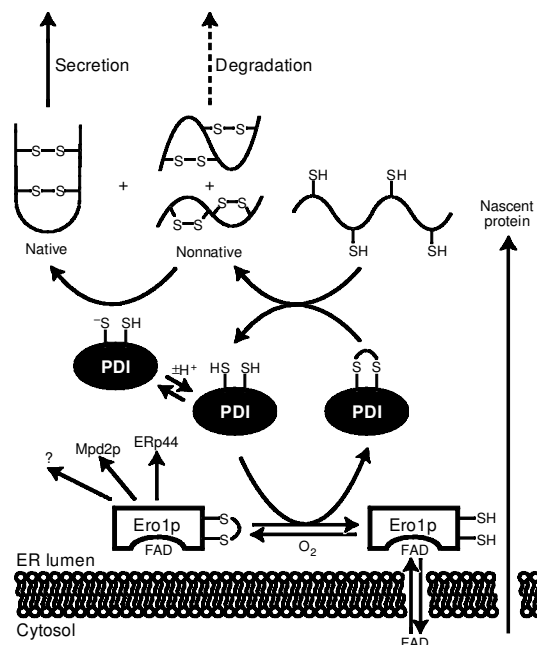
## DITHIOL OXIDATION

The process of native disulfide formation involves both the oxidation of dithiols to disulfides and the rearrangement of nonnative to native disulfide bonds. PDI is involved in both of these steps, but its contribution to each is not yet fully understood. In recent years, much focus has been on the oxidative role of PDI in the cell (24, 58, 84).

Ero1p [and its human homologues Ero1- $\alpha$  and Ero1-L $\beta$  (4, 66)] is a membrane-associated ER protein that was discovered in a screen for proteins that confer resistance to dithiothreitol (DTT) when overproduced or cause sensitivity to DTT when altered (22, 70). Ero1p is an essential enzyme in yeast that oxidizes proteins in the ER (22, 70). Ero1p is specific in its choice of substrates, interacting with only a few proteins (23). Most importantly, Ero1p and PDI form a mixed disulfide *in vivo* (23). Ero1 in mammalian cells specifically oxidizes the C-terminal active site of PDI during the PDI-catalyzed retrotranslocation of cholera toxin (82). Mutational analysis of Ero1p has suggested that a CXXXXC motif is responsible for oxidizing PDI and other substrates, whereas a distinct CXXC motif reoxidizes its catalytic site (24). This finding, combined with evidence that PDI is involved in intermolecular disulfide bonds with its substrates (23, 59), suggests a pathway for the formation of disulfide bonds in newly synthesized proteins (Fig. 3). In the lumen of the ER, flavin adenine dinucleotide (FAD)-bound Ero1p oxidizes PDI (84), which accumulates in its reduced form in the absence of Ero1p. Oxidized PDI then transfers oxidizing equivalents from Ero1p to reduced substrate proteins. Ero1p is then reoxidized by molecular oxygen (83). The oxidative folding cycle, is regulated by levels of free FAD in the ER (83).

Contrary to previous assumptions, studies with Ero1p have revealed that the majority of PDI in the ER of *S. cerevisiae* exists in the oxidized state (23). To catalyze disulfide isomerization, PDI must be in its reduced form. The ER, which has  $E_{\text{solution}} = -0.18$  V (37), seems to be the optimum environment for PDI to carry out oxidative protein folding. With  $E^{\circ'} = -0.18$  V, PDI at equilibrium in the ER would exist as an equimolar mixture of reduced and oxidized forms (53). The finding that most PDI in the ER of yeast is in the oxidized state suggests, however, that the majority of the enzyme is involved in disulfide formation rather than isomerization.

The situation appears to be quite different for human PDI. In three human cell lines (HeLa, COS, and U937), virtually all of the cellular PDI is reduced (58). The oxidized form of PDI only becomes detectable upon exposure of the cells to high levels of DTT. Perhaps the redox system in human cells is more complex than is that in *S. cerevisiae* cells. For example, additional PDI and Ero homologues in human cells could allow PDI to be a specific catalyst of isomerization, as other enzymes take up the slack in oxidation.



**FIG. 3. The pathway of native disulfide formation in the lumen of the ER.** FAD-bound Ero1p (84) (and presumably Ero1- $\alpha$  and Ero1-L $\beta$  in humans) specifically oxidizes PDI as well as ERp44 (human cells), Mpd2p (yeast), and perhaps other proteins (1, 23). Ero1p uses molecular oxygen to reoxidize itself for further folding cycles (83). Oxidized PDI catalyzes the formation of disulfide bonds in newly synthesized proteins; the thiolate form of reduced PDI catalyzes the isomerization of nonnative disulfide bonds (49). Proteins that do not achieve the native state are degraded rather than secreted. For simplicity, only one of the two active sites of Ero1p and PDI is shown.

## BALANCING ISOMERIZATION AND OXIDATION

Although the majority of PDI in *S. cerevisiae* appears to be devoted to catalyzing the formation of disulfide bonds, isomerization of disulfide bonds limits the rate of native disulfide formation. This task is not accomplished by another enzyme in the absence of PDI. Yet other proteins fulfill the oxidative role of PDI in *pdi1Δ* yeast, perhaps via the yeast unfolded protein response pathway (8, 49, 63).

Only a small amount of isomerization activity is necessary for viability. The isolated a and a' domains of rat PDI possess isomerization activity that is only 8.5% and 14%, respectively, that of wild-type PDI (12). These values agree well with the finding that having only a single reactive cysteine residue in the full-length protein results in a rate of sRNase A folding that is 10–12% lower than that of wild-type PDI (85). Even with this low activity, the isolated a and a' domains are able to rescue *pdi1Δ S. cerevisiae* with growth rates that are comparable to those with wild-type PDI (98). When overproduced, Eug1p is able to suppress a PDI deficiency in yeast, even though the *in vitro* isomerase activity of this CXXS enzyme is poor (62, 80). Trx variants that complement a PDI deficiency are likely to be largely oxidized in the ER, and hence poor catalysts of isomerization (Table 2) (8, 10). Taken to-

gether, the available data indicate that a minimal amount of reactive thiolate is required to initiate isomerization reactions in misfolded proteins in the ER.

The physical properties of PDI are ideal for catalysis of disulfide formation and isomerization in the ER of eukaryotic cells (Fig. 3). In contrast, bacteria have evolved two distinct systems to accomplish these tasks. In bacteria, the DsbA/DsbB system is responsible for the formation of disulfide bonds, whereas the DsbC/DsbD system accomplishes nonnative disulfide isomerization (16). DsbB is the oxidant for DsbA that can accept electrons from reduced substrates. If substrates form nonnative disulfide bonds, the periplasmic DsbC protein is responsible for their rearrangement. DsbC is maintained in a reduced state by the membrane-bound protein, DsbD. The function of bacterial oxidoreductases is discussed further in the accompanying review by Ortenberg and Beckwith (64).

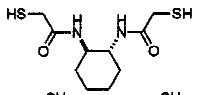
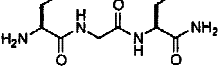
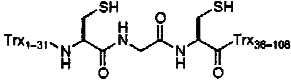
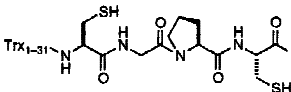
## SMALL-MOLECULE MIMICS OF PDI

The primary determinants of isomerization efficiency are an  $E^{\circ'}$  near  $-0.18$  V and a thiol  $pK_a$  near 7.0. By using these criteria, it should be possible to design small-molecule mimics of nature's enzymic isomerase. Such a small-molecule "foldase" would have a number of practical applications. Large-scale production of secretory proteins in *E. coli* is often complicated by aggregation or proteolysis associated

with unfavorable conditions for disulfide formation in the bacterial cytosol (27, 55). Insoluble protein aggregates must be solubilized and folded *in vitro* in an appropriate redox buffer. Glutathione is often used for this task, but is not an efficient catalyst of oxidative protein folding. PDI is not a practical catalyst for large-scale protein production due to the high cost of its production, its instability, and the necessity to separate it from a target protein. An effective small-molecule catalyst would be desirable for oxidative protein folding *in vitro*, as well as for addition directly to cell cultures to improve heterologous protein production (74).

Although only one cysteine residue is required for catalysis of disulfide isomerization, the presence of two cysteine residues in the active site enhances the overall effectiveness of PDI in the formation of native disulfide bonds (62, 85). Hence, it is reasonable to assume that a small-molecule dithiol would be a more active catalyst of protein folding than would an analogous monothiol. This assumption has been confirmed by experiments. The dithiol ( $\pm$ )-*trans*-1,2-bis(mercaptoacetamido)cyclohexane (BMC or Vectrase<sup>TM</sup>-P) has thiol  $pK_{a1} = 8.3$  and  $pK_{a2} = 9.9$  and  $E^{\circ'} = -0.24$  V (Table 4) (96). These values approach those of the CXXC motif in PDI, suggesting that this molecule may be efficient in catalyzing disulfide isomerization. Indeed, BMC increases both the rate of reactivation of sRNase A and the final yield of native RNase A over that attainable with glutathione (96). In addition, BMC (a dithiol) shows an increase in activity and recovery over that with *N*-methylmercaptoacetamide (a monothiol) (96). The presence of two sulfhydryl groups allows rescue from trapped

TABLE 4. PROPERTIES OF SMALL-MOLECULE DITHIOL CATALYSTS OF DISULFIDE ISOMERIZATION

Molecule	Structure	First $pK_a$	$E^{\circ'}$ (V)	Specific Activity (U/mol) <sup>a</sup>
BMC		8.3 <sup>b</sup>	$-0.240^b$	56 <sup>c</sup>
CGC		8.7 <sup>d</sup>	$-0.167^d$	132 <sup>c</sup>
CGC Trx		ND	$\geq -0.200^e$	3,300 <sup>c</sup>
CGPC Trx		7.5 <sup>f</sup>	$-0.270^g$	0 <sup>c</sup>

ND, not determined.

<sup>a</sup>One unit (U) of reduced catalyst activates 1 mmol of sRNase A per min in 0.1 M Tris-HCl buffer, pH 7.6, containing EDTA (1 mM).

<sup>b</sup> $pK_a$  value was determined by ultraviolet spectroscopy;  $E^{\circ'}$  value was determined by equilibration with  $\beta$ -mercaptoethanol/ $\beta$ -hydroxyethylthiol (96).

<sup>c</sup>Data from ref. (95).

<sup>d</sup> $pK_a$  value was determined by ultraviolet spectroscopy;  $E^{\circ'}$  value was determined by equilibration with  $\beta$ -mercaptoethanol/ $\beta$ -hydroxyethylthiol (95).

<sup>e</sup>Determined from the equilibrium constant for the thioredoxin reductase-catalyzed reaction with NADPH/NADP<sup>+</sup> (95).

<sup>f</sup>Determined by <sup>13</sup>C-NMR spectroscopy for the state in which Asp26 and Cys35 are protonated (10).

<sup>g</sup>Determined from the equilibrium constant of the thioredoxin reductase-catalyzed reaction with NADPH/NADP<sup>+</sup> (48).

mixed disulfides between catalyst and substrate, resulting in greater recovery of active RNase A and an increased rate of isomerization. An immobilized analogue of BMC has similar attributes and provides another strategy for preparative protein folding *in vitro* (97).

BMC is also an effective disulfide isomerase *in vivo*. *S. pombe* acid phosphatase is a 30-kDa homodimer with eight disulfide bonds (74, 93). *S. cerevisiae* cells grown in the presence of BMC (0.1 g/L) secrete threefold more acid phosphatase, an increase equivalent to that achieved with 15-fold overproduction of PDI (96). Likewise, the presence of BMC (2  $\mu$ g/L) in an *E. coli* culture medium increases the yield of proinsulin, which has three disulfide bonds, by 60% (92). These data indicate that a small-molecule mimic of PDI can enhance the yield of heterologous protein production in both eukaryotic and prokaryotic systems.

Aromatic thiols are also efficient small-molecule foldases. Aromatic thiols have low  $pK_a$  values ( $pK_a = 3-7$ ) compared with their aliphatic counterparts ( $pK_a = 7-11$ ) and are more reactive for thiol-disulfide interchange reactions at physiological pH (90, 91). Of particular interest is 4-mercaptobenzenesulfonate, which has a  $pK_a = 6.6$ , close to that of active-site thiols in PDI (17). This reactive monothiol is able to catalyze the reactivation of sRNase A at a rate that is five- to sixfold higher than that of glutathione (31). Further rate enhancement could be attainable with an aromatic dithiol.

Other design strategies for small-molecule foldases are based more literally on the active site of PDI. Linear CXXC peptides that mimic the active sites of a variety of oxidoreductases exhibit reduction potentials that do not reflect the enzyme from which they were derived. The reduction potential of the disulfide bond in the W-CGPC-KHI peptide, for example, is 70 mV lower than that of native CGPC Trx (78). Varying the -XX- sequence of linear CXXC octapeptides results in minimal changes in its redox properties with  $E^{\circ'}$  values [calculated from equilibrium constants and  $E^{\circ'}$  of -0.252 for glutathione (52)] in the range of -0.220 V to -0.200 V (78).

On the other hand, by restricting the conformational freedom of the active-site motif in a cyclic hexapeptide, CXXC-containing peptides can be more efficient oxidative protein folding catalysts (5). Three cyclic peptides, with sequences corresponding to glutaredoxin reductase, Trx, and PDI, show increasing activity and yield of native protein in a rRNase A assay. The enhancement in catalytic efficiency corresponds to a decrease in thiol  $pK_a$  and an increase in  $E^{\circ'}$ .

An alternative to changing the identity of intervening residues in a CXXC-containing peptide is to change the number of intervening residues in a C(X)<sub>n</sub>C-containing peptide. To obtain an effective isomerase, it is desirable to have a fairly unstable disulfide bond. CXC peptides, with only one intervening residue, form 11-membered disulfide-bonded rings and typically have  $E^{\circ'}$  values that are 30-40 mV lower than CXXC peptides (43, 102). A CGC peptide is a promising candidate for a small-molecule isomerase (95). Although the  $pK_a$  of the reactive thiolate of CGC is higher than that of BMC, the reduction potential matches that of the PDI active site (Table 4). The CGC peptide is more efficient at folding sRNase A than is BMC. Even without imposing conformational constraints, a linear CGC is an effective catalyst of disulfide isomerization due to its favorable redox properties.

Small-molecule dithiols can act as isomerases, yet their activities lie far below that of PDI. Although the presence of a CXXC (or CXXS) motif is the only absolute requirement for isomerase activity, the protein scaffold provided by PDI increases the catalytic efficiency of the motif (87). Not only does the protein scaffold form a conformationally constrained active site that serves to regulate its redox properties, it also provides a means of noncovalent interaction. In addition to covalent rearrangements, catalysis of disulfide isomerization relies on noncovalent interactions between PDI and its substrates. The b' domain of PDI provides a binding site for unfolded proteins (7, 44, 61). Mutagenesis studies of individual PDI domains have shown that when combined with the a' domain, the b' domain improves the rate of bovine pancreatic trypsin inhibitor (BPTI) isomerization by 75% over that with the a' domain alone (14). Further, full-length PDI is seven- to 12-fold more active in the folding of sRNase A than is the a or a' domain alone (12).

Can an improved isomerase—one that combines the benefits of covalent and noncovalent interactions—be obtained by inserting a chemical catalyst into the context of a stable protein that has affinity for unfolded proteins? To answer this question, a CGC motif has been placed at the active site of Trx. Wild-type CGPC Trx has low activity in the catalysis of native disulfide formation due to the stability of its active-site disulfide bond (68). The disulfide bond destabilization imposed by deletion of a proline residue in the active site endows reduced CGC Trx with the ability to reactivate sRNase A *in vitro* (Table 4) (95).

The development of chemical catalysts that mimic the properties of PDI will be beneficial to both biotechnology and biomedicine. The production of eukaryotic proteins in *E. coli* often requires additional steps to obtain properly folded protein (55). Current techniques are neither time- nor cost-efficient. Small-molecule catalysts like BMC and CGC will provide better options for native disulfide formation during heterologous protein expression and purification from inclusion bodies. Defects in protein folding have been implicated in several diseases (18). For example, Alzheimer's disease coincides with a down-regulation of the ER stress response (38, 67), in which PDI is a key player (19). A small-molecule mimic of PDI could thus be a useful chemotherapeutic. Conversely, the catalytic activity of cell-surface PDI is necessary for the entry of HIV-1 virus into T lymphocytes (2, 26, 56). Hence, cell-surface PDI is a valid target for inhibitor development.

## NEED FOR MORE MECHANISTIC UNDERSTANDING

What is the precise chemical mechanism by which PDI achieves disulfide formation and isomerization? What is the substrate specificity of PDI, and its molecular basis? How is PDI able to balance its role as a disulfide isomerase and dithiol oxidase? Two major obstacles hinder researchers seeking answers to these and related questions. First, the lack of a three-dimensional structure of intact PDI makes it difficult to obtain a clear picture of the active-site residues within the

enzyme, as well as between the enzyme and its substrates. Second, substrates now available for studying disulfide isomerization are too complex to delineate the details of the chemical mechanism.

The structures of individual a and b domains of PDI are known (Fig. 1b) (41, 42) and provide evidence for structural similarity between all four of the major domains. It is not yet understood either how the four domains interact with each other or how the two active sites communicate with each other. At saturating concentrations of substrate, the two active sites play different roles. The N-terminal active site is responsible for most of the catalytic activity, whereas the C-terminal active site contributes more to substrate binding (54). At substrate concentrations near the value of  $K_M$  (where the rate is half-maximal), the two active sites are equally efficient catalysts and function independently (86). In contrast, communication between the two active sites plays an integral role in catalysis by *E. coli* DsbB (40) and *S. cerevisiae* Ero1p (24).

Structures of PDI homologues provide some intriguing proposals for the arrangement of individual PDI domains. CVWC Trx crystallizes in a dimer-like structure (76). This dimer is connected through a continuous  $\beta$ -sheet across both monomers and interlocked  $\alpha$ -helices that cap the  $\beta$ -strands. Similarly, the structure of a disulfide oxidoreductase from *Pyrococcus furiosus* contains two CXXC-containing Trx-like units that are joined by a continuous  $\beta$ -sheet (73). In both of these examples, adjacent Trx-like units are arranged colinearly.

In PDI, the two CXXC motifs do not form disulfide bonds of equivalent stabilities. Circular dichroism studies of isolated catalytic domains suggest that the a' domain contains a very unstable disulfide bond, whereas the disulfide bond of the a domain is relatively stable (12). Similarly, the structure of the *P. furiosus* enzyme reveals differences between the stabilities of the two active-site disulfide bonds (73). The more N-terminal disulfide bond is highly flexible due to conformational strain imposed by unfavorable dihedral angles. This flexibility results in a destabilized disulfide bond. The more C-terminal disulfide bond is more stable and has dihedral angles resembling that in other Trx homologues. The difference in stability between the two active-site disulfide bonds in PDI could reflect a difference in their physiological roles.

The structure of DsbC provides useful information on that of PDI (57). DsbC is the enzyme responsible for catalyzing disulfide isomerization in the bacterial periplasm (72, 100). DsbC is a dimeric protein, and, like the enzymes described above, its monomers are joined through a continuous  $\beta$ -sheet. Each monomer is composed of a catalytic N-terminal domain and a C-terminal dimerization domain. This arrangement is similar to the arrangement of domains in PDI in which the a and a' domains are catalytic and the b and b' domains serve another role. In the DsbC crystal structure (57), the N- and C-terminal domains are connected by a hinged  $\alpha$ -helix that allows for flexibility of the catalytic domain. Between the two active sites is a broad uncharged cleft that can accommodate the nonspecific binding of misfolded substrate proteins. The flexibility provided by the hinged region allows for variation in the size of the cleft to allow binding of large or small substrate proteins. This structure provides a model for how the domains of PDI might interact to allow the nonspecific binding of substrates.

The structure and function of DsbC in a complex with the N-terminal domain of DsbD (DsbD $\alpha$ ) provides insight on how a disulfide isomerase interacts with a protein substrate (30, 33). DsbD $\alpha$  binds in the cleft of DsbC, causing conformational changes in both the substrate and catalyst. Both active sites of DsbC contact DsbD $\alpha$  cysteine residues. The primary noncovalent interactions are between hydrophobic or uncharged polar groups.

Even with more structural information, a proper substrate for detailed mechanistic work is essential. Currently, the most common *in vitro* substrates for studying catalysis by PDI are RNase A and BPTI (11), neither of which is ideal. RNase A is a 124-residue protein with eight cysteine residues that can be randomly oxidized under denaturing conditions to give sRNase A, a mixture of up to 105 ( $= {}_8C_8 \times 7 \times 5 \times 3$ ) distinct fully oxidized species, only one of which is native. Moreover, RNase A can form 764 ( $= {}_8C_8 \times 7 \times 5 \times 3 + {}_8C_6 \times 5 \times 3 + {}_8C_4 \times 3 + {}_8C_2 + {}_8C_0$ ) different oxidized and reduced species altogether. BPTI, a 58-residue protein, is a less complex substrate with only six cysteine residues. Still, BPTI can form 15 ( $= {}_6C_6 \times 5 \times 3$ ) distinct fully oxidized species.

Simple peptides have been used to study disulfide formation and reduction by PDI. A 28-residue peptide, which is based on the sequence of BPTI but with only one disulfide bond, is a substrate for catalysis of oxidation by PDI in the presence of glutathione (13). A fluorescently labeled peptide of seven residues and one disulfide bond has been used to study the oxidation and reduction activities of PDI (88). Recently, a variant of green fluorescent protein with a redox-sensitive disulfide bond has been developed as a tool to monitor disulfide bond formation in living cells (65).

Likewise, a simple substrate could be used to characterize disulfide isomerization by PDI in an *in vitro* assay. The ideal substrate would contain only two disulfide bonds and thus only three fully oxidized forms. To be a substrate for characterizing disulfide isomerization activity, the two disulfide bonds in the native form must be both stable under the conditions of a typical folding assay ( $E'_{\text{solution}} = -0.18$  V) and more stable (higher  $E^\circ$ ) than those in the other two fully oxidized forms. A continuous folding assay could be used with such a substrate. For example, by modifying the substrate to contain an appropriate donor and acceptor, fluorescence resonance energy transfer (FRET) could be used to monitor any conformational change that accompanies disulfide isomerization. Unlike the discontinuous assays currently available, a continuous folding assay would permit rapid, detailed analysis of the chemical and kinetic mechanism by which PDI catalyzes the isomerization of nonnative disulfide bonds.

## CONCLUSIONS

Although disulfide bonds are responsible for stabilizing the native, active structure of many secretory proteins, the formation of the correct disulfide pairs is not always a simple task. As the number of cysteine residues in a protein increases, the chance of forming nonnative disulfide bonds also increases. PDI is an essential enzyme in eukaryotes that prevents the accumulation of nonnative and hence inactive

species. By providing a reactive thiolate, PDI is able to catalyze disulfide isomerization, its essential function. With help from Ero1p, PDI is also able to play an important role in oxidizing newly synthesized proteins. An interesting topic for future research will be to determine how PDI is able to balance its roles as an isomerase and oxidase in the ER. Understanding the mechanism of disulfide isomerization will allow further development of small-molecule and enzymic isomerases that can be used to improve heterologous protein production and be adapted for biomedical purposes. Although the reduction potential and  $pK_a$  are good indicators of thiolate reactivity, they do not fully explain the differences in effectiveness of catalysts of disulfide isomerization. A three-dimensional structure of the complete PDI protein would be invaluable in understanding how PDI recognizes and interacts with its substrates, and converts them to native (active) proteins in the ER. Further mechanistic studies will also be aided by the development of simple two-disulfide substrates that could be used to test a variety of catalysts in a continuous disulfide isomerization assay.

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## ABBREVIATIONS

BMC, ( $\pm$ )-*trans*-1,2-bis(mercaptoacetamido)cyclohexane; BPTI, bovine pancreatic trypsin inhibitor; DsbA, periplasmic protein thiol:disulfide oxidoreductase from *Escherichia coli*; DsbD $\alpha$ , N-terminal domain of DsbD; DTT, dithiothreitol; ER, endoplasmic reticulum; FAD, flavin adenine dinucleotide; PDI, protein disulfide isomerase; proCPY, procarboxypeptidase Y; RNase A, ribonuclease A; rRNase A, reduced RNase A; sRNase A, scrambled RNase A; Trx, thioredoxin.

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