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21

Protein Disulfide Isomerase: Cellular Enzymology of the CXXC Motif

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I. DISULFIDE BONDS IN THE EUKARYOTIC CELL

Disulfide bonds between the thiol groups of cysteine residues stabilize the native structures of many proteins. The pathway for the formation of native disulfide bonds is complex (1,2). This complexity arises because the thiol groups and disulfide bonds of proteins can undergo three distinct chemical reactions: dithiol oxidation, disulfide bond reduction, and disulfide bond isomerization (3). Of the three, only disulfide bond isomerization does not require the concomitant reduction or oxidation of another molecule (Fig. 1).

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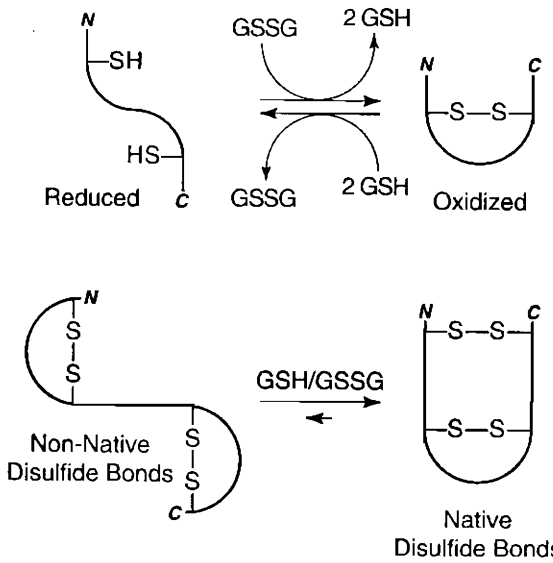

Fig. 1 Reactions catalyzed by PDI in vitro. The enzyme catalyzes the oxidation of dithiols and reduction of disulfide bonds (top), and the isomerization of disulfide bonds (bottom).

The tendency of two thiols to form a disulfide bond or of an existing disulfide bond to be broken depends on the reduction potential of the environment (3). The cytosol of eukaryotic cells has a low reduction potential of $E^{\circ\prime} = -0.230$ V (4).* In comparison, the endoplasmic reticulum (ER) has a high reduction potential of $E^{\circ\prime} = -0.172$ to -0.188 V (4). These values largely dictate the redox state of disulfide bonds in cellular proteins. In a typical unfolded protein two thiols have an effective molarity near 50 mM relative to two molecules of reduced glutathione (6,7). Using the reduction potential of oxidized glutathione [$E^{\circ\prime} = -0.252$ V (5)] and the Nernst equation, the reduction potential of a typical disulfide bond in an unfolded protein is calculated to be $E^{\circ\prime} = -0.22$ V. This value is between that of the cytosol and the ER, indicating that the formation of disulfide bonds in unfolded proteins is favored in the ER but not in the cytosol. In contrast, disulfide bonds in folded proteins have reduction potentials as low as $E^{\circ\prime} = -0.45$ V (8). Thus, reduction potentials tend to increase in the order: folded protein < cytosol < unfolded protein < ER. This order suggests that disulfide bonds form in unfolded proteins as they are being translocated into the ER.

*Different values for the $E^{\circ\prime}$ of oxidized glutathione have been used to calculate biochemical reduction potentials (5). The values quoted herein are uncorrected for this inconsistency, which has little qualitative impact.

Some of these bonds will be those found in the properly folded protein, but others will be nonnative (9).

II. PROPERTIES OF PROTEIN DISULFIDE ISOMERASE

Eukaryotic cells are now known to contain an ensemble of proteins that orchestrate the proper folding of other proteins (10). The search for an enzymic catalyst of oxidative protein folding began more than 30 years ago. In 1963, an enzyme capable of catalyzing the reactivation of reduced ribonuclease A was discovered in microsomal preparations from rat liver (11) and from chicken and pigeon pancreas (12). This enzyme was eventually named protein disulfide isomerase (PDI; EC 5.3.4.1). In 1964, it was discovered that the rate of thiol disappearance during the air oxidation of reduced ribonuclease A is independent of PDI but that the rate of reactivation relies on the enzyme (13). These results indicated that PDI does not catalyze the air oxidation of dithiols. Rather, air oxidation involves the uncatalyzed formation of nonnative disulfide bonds followed by the PDI-catalyzed isomerization to the disulfide bonds in the native protein (13). This discovery, along with the observation that PDI enhances the rate of reactivation of both reduced ribonuclease A and reduced lysozyme (14), led Anfinsen and coworkers to favor "the hypothesis that the enzyme is a general and nonspecific catalyst for disulfide interchange in proteins containing disulfide bonds" (13).

PDI is a 57-kDa protein that constitutes approximately 2% of the protein in the ER (15). The enzyme has been shown to catalyze each of the reactions shown in Fig. 1—the oxidation of dithiols, and the reduction and isomerization of disulfide bonds—by the in vitro assays listed in Table 1. The products of redox catalysis by PDI depend on the dithiol–disulfide reduction potential of the substrate and the solution.

Early work on PDI implicated one or more cysteine residues as being necessary for its enzymatic activity. Carboxymethylation or carbamoylmethylation of PDI caused irreversible inactivation (25,26). In addition, PDI was shown to be inhibited by arsenite or Cd^{2+}, behavior diagnostic of enzymes with active-site dithiol groups (27,28).

In 1985, Rutter, Roth, and coworkers verified the existence of the inferred cysteine residues by cloning and sequencing the cDNA that codes for rat PDI (29). The cDNA sequence revealed that the rat enzyme is synthesized as a 528-residue precursor that contains a 19-residue signal sequence. The mature enzyme contains 509 residues, resulting in a molecular mass of 56.8 kDa (29,30). Rat PDI consists of two pairs of homologous regions: amino acid residues 9–90 (region a) and 353–431 (region a'), and amino acid residues 153–244 (region b) and 256–343 (region b'). Regions a and a' each contain an active site with the sequence WCGHCK, which is homologous to the single active site in *Escherichia coli* thioredoxin (31). The C terminus of PDI ends with the

Table 1 Assays for PDI Activities

Activity	Substrate
Dithiol oxidation	Reduced ribonuclease A (22)
	Reduced lysozyme (14,23)
	Reduced bovine pancreatic trypsin inhibitor (16,24)
	Reduced human gonadotropic hormone (18)
	Reduced Fab fragment (20)
	Reduced synthetic peptide (17)
Disulfide bond reduction	Insulin (21)
	Fab fragment (20)
	Synthetic peptide (17)
Disulfide bond isomerization	Scrambled ribonuclease A (19)
	Partially reduced pancreatic trypsin inhibitor (24)

sequence KDEL, which has been implicated as the signal for retention of a protein in the ER of mammalian cells (32). cDNAs encoding PDI have also been sequenced from human (33), mouse (34), and bovine (35) tissues.

In 1991, the gene that codes for yeast PDI was isolated during the yeast genome sequencing project (36). Yeast PDI is a 522-residue protein that contains a C-terminal HDEL sequence—the yeast ER retention signal (37). Yeast PDI also contains five consensus N-glycosylation sites, each of which appears to be modified in the protein found in wild-type cells. The yeast PDI amino acid sequence shares 29% identity and 44% similarity with mammalian PDI. Most significantly, the cloning of the gene for yeast PDI enabled Scherens and coworkers to demonstrate that the *PDI1* gene is essential for the viability of *S. cerevisiae cells* (36). Recent results from our laboratory show that PDI is more important for spore germination than cell division (38).

Still, the question remained: What cellular process is impaired by the absence of PDI? The enzyme catalyzes dithiol oxidation as well as disulfide bond reduction and isomerization (39). On the other hand, PDI can bind to peptides (40,41). Moreover, PDI is the β subunit of prolyl 4-hydroxylase, an $\alpha_2\beta_2$ tetramer that catalyzes the formation of 4-hydroxyproline residues in collagen strands (42,33). PDI is also a subunit of the heterodimeric microsomal triglyceride transfer protein (43,44). Neither of these complexes is known to be present in yeast cells.

Two other functions have been erroneously ascribed to PDI. Although it has some affinity for thyroid hormones and has been deemed a thyroid hormone–binding protein (45), PDI is not involved in thyroid hormone metabolism (46). Similarly, PDI has an affinity for peptides, including glycosylated peptides (47), but it is not the glycosylation site–binding protein responsible for core glycosylation of nascent chains in the ER (48).

III. ROLE OF PROTEIN DISULFIDE ISOMERASE IN THE CELL

PDI is the most efficient known catalyst of oxidative protein folding (49,50). Surprisingly, the catalysis by the PDI subunit is unnecessary for the function of either prolyl 4-hydroxylase (51,52) or the microsomal triglyceride transfer protein (A. D. Attie, personal communication). Still, creation of a protein catalyst is demanding and unlikely to be without purpose.

Studies based on the complementation of *PDI1* null mutants of *Saccharomyces cerevisiae* have provided clues as to the essential role of PDI in eukaryotic cells. In 1992, Tachibana and Stevens showed that overexpression of the *EUG1* gene, which codes for an ER protein with CLHS and CIHS sequences, allows *pdi1*Δ cells to grow (53). This important finding showed that the C-terminal cysteine residue of the CXXC motif is not essential to eukaryotic cells.

In 1993, LaMantia and Lennarz used genetic and enzymatic data to claim that PDI is not needed for the catalysis of disulfide bond isomerization in *S. cerevisiae* (54). These workers found that cDNA coding for CLHS/CIHS PDI (which mimics Eug1p) complements *pdi1*Δ *S. cerevisiae* but does not catalyze the oxidation of dithiols. This observation spawned the widespread notion that PDI acts in vivo primarily as a chaperone or even as an "antichaperone" (23,55–57). These data only show, however, that PDI is unnecessary for the *formation of* disulfide bonds. They do not address the role of PDI in the *isomerization* of existing disulfide bonds (Fig. 1).

Is the isomerization activity of PDI essential for the viability of *S. cerevisiae*? We answered this question by testing the ability of mutant enzymes both to replace PDI in *S. cerevisiae* and to catalyze *each* of the activities in Fig. 1 (58). In this work, rat PDI was used to avoid complications arising from the extensive glycosylation of yeast PDI. Double mutants of rat PDI were created in which either the N-terminal or C-terminal cysteine residue in each active site is replaced by a serine. The results of genetic analyses indicated that a cDNA for wild-type or CGHS PDI is able to complement *pdi1*Δ *S. cerevisiae*. In contrast, a cDNA for SGHC PDI is unable to compensate for this deficiency.

We then produced the wild-type and mutant PDIs using a yeast expression system (59) and assayed the three enzymes for each enzymatic activity demonstrated by PDI (Fig. 1). The results of these assays are shown in Fig. 2. In comparison to wild-type (i.e., CGHC) PDI, SGHC and CGHS PDI have negligible dithiol oxidation activity (measured by an increase in activity of reduced ribonuclease A) and negligible disulfide reduction activity (measured by the cleavage of porcine insulin). In contrast, CHGS PDI has isomerization activity (measured by an increase in activity of scrambled ribonuclease A) comparable to that of the wild-type enzyme. SGHC PDI has negligible isomerization activity. The essential function of PDI is therefore enzymic but does not relate to the

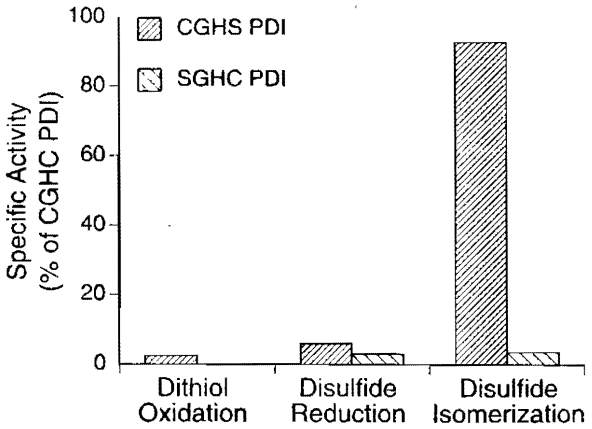

Fig. 2 Catalytic activities of CGHS and SGHC PDI. The data are shown relative to those of the CGHC (i.e., wild-type) enzyme (58).

net formation of protein disulfide bonds. Rather, the role of PDI in vivo is to act as a "shufflease"—a catalyst of disulfide bond isomerization (Fig. 1, bottom).

The properties of the Eug1p protein support this conclusion. Overexpression of the *EUG1* gene complements *pdi1*Δ *S. cerevisiae*. Wild-type Eug1p is analogous to the shufflease mutant of PDI in that each active site contains only the N-terminal cysteine residue (53). Thus, the essential functional atom in the CXXC motif is the sulfur of the N-terminal cysteine residue.

IV. MECHANISM OF CATALYSIS BY THE CXXS MOTIF

Catalysis of disulfide bond reduction or thiol oxidation depends on the redox environment (3). In contrast, during catalysis of disulfide bond isomerization, the substrate does not undergo a net change in oxidation state (Fig. 1). Thus, neither a redox-active catalyst nor a redox-active buffer is essential for catalysis. The simplest mechanism for catalysis of an isomerization reaction by a PDI shufflease, a redox-inactive catalyst, is shown in Fig. 3. This mechanism begins with the attack of a thiolate ion on a protein disulfide, forming a mixed disulfide (60). 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. Testing this mechanism will require the development of a simple, well-defined substrate (unlike those in Table 1) for the isomerization reaction.

Disulfide bond isomerization is driven by the search for the most stable conformation of the substrate protein. Still, PDI (or shufflease) could continue to attack the native disulfide bonds in a folded protein ad infinitum. Why does

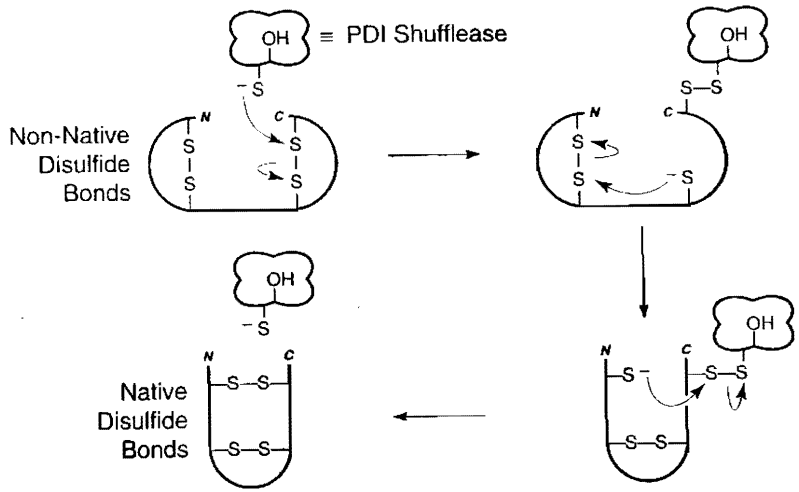

Fig. 3 Simplest mechanism for catalysis of disulfide bond isomerization by shufflease (i.e., CGHS PDI).

PDI stop? As a catalyst, PDI does not change the relative free energy of the unfolded and native conformations of a protein. Yet the effective molarities [EM, or K_{ox} (3)] of the thiols in the unfolded and native conformation differ dramatically. In an unfolded protein, the EMs of the thiols are approximately 50 mM relative to the thiols in reduced glutathione (6,7). In a folded protein, the EMs of the thiols that form native disulfide bonds are often 10^3–10^7 M (8). In contrast, using the Nernst equation and the reduction potential of PDI (61), the

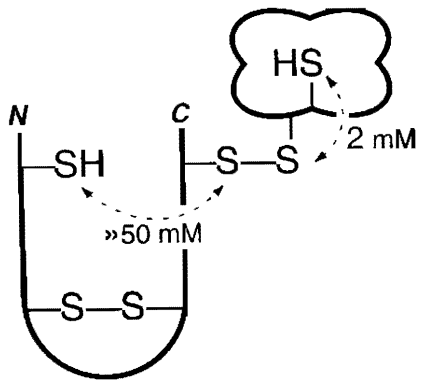

Fig. 4 Effective molarities in the mixed disulfide formed by attack of PDI on a disulfide bond in a native protein.

EM of the active-site thiols in PDI is calculated to be 2 mM. Thus, the EM of the thiols that form the native disulfide bonds of a folded protein is likely to be much greater than that in the active site of PDI. This analysis suggests that although PDI or shufflease can attack a native disulfide bond to form a mixed disulfide, the native disulfide bond will have an overwhelming propensity to reform as is implied in Fig. 4.

V. PROPERTIES OF THIOREDOXIN

PDI is homologous to thioredoxin (Trx), a 12-kDa cytosolic reducing agent for ribonucleotide reductase and other proteins (62). Trx does not possess the multiplicity of nonredox functions exhibited by PDI (33,42,44). For example, Trx does not bind to a peptide that was used to identify a peptide binding region in PDI (55). Still, the amino acid residues that surround the CXXC motifs of PDI and Trx are those that are most conserved between the two proteins (29).

The presence of Trx in all types of organisms (64) argues for its early evolutionary appearance. The discovery of other proteins with Trx domains suggests that these domains were recruited for a specific purpose. For example, PDI has two Trx domains. The gene for ERp72, a murine ER protein with three CXXC motifs, can complement *pdi1*Δ yeast (65). Likewise, overexpression of *MPD1*, a yeast gene encoding an ER protein with a single CXXC sequence, complements *pdi1*Δ yeast (63). DnaJ, an *E. coli* chaperone, has four CXXC sequences and catalyzes each reaction in Fig. 1 (66). DsbA, a protein of the *E. coli* periplasm, has a CXXC motif and was discovered because of its ability to rescue *E. coli* mutants defective in disulfide bond formation (67). DsbA shares no sequence similarity with *E. coli* Trx apart from the active site, but the two proteins do have similar three-dimensional structures (68).

Overall, PDI and Trx share approximately 30% amino acid identity and have similar active sites: CGHC in each Trx domain of PDI (29) and CGPC in Trx (69). Unlike PDI from any organism, *E. coli* Trx is a small, well-characterized protein (62). A crystalline structure of the oxidized form is known to 1.68 Å resolution (70), and solution structures are known of both the oxidized and the reduced forms (71). Although *PDI1* is essential for the viability of *S. cerevisiae* (36), *trxA* is not essential for *E. coli* (72).

The active sites of PDI and Trx are similar. The results of chemical modification studies and pK_a determinations on PDI are parallel to those on Trx (73), suggesting that the reactivities of the active sites are similar. In addition, PDI is a substrate for thioredoxin reductase, which suggests that the three-dimensional structures of the active sites are alike. In native Trx, the most pronounced deviation from an almost spherical surface is a protrusion formed by residues 29–37, which includes the active site. The thiol of Cys32, which has a low pK_a, is exposed to the solvent, but the thiol of Cys35 is recessed.

VI. THIOREDOXIN AS A SUBSTITUTE FOR PROTEIN DISULFIDE ISOMERASE

We used *E. coli* Trx to reveal which domains of PDI are essential in vivo and which properties of the CXXC motif are most important to eukaryotic cells (74). Trx is normally found in the cytosol. To illuminate the function of the CXXC motif in eukaryotic cells, *E. coli* Trx was targeted to the ER of *S. cerevisiae* by fusing it to the C-terminus of the α-factor pre–pro segment (75). Trx was retained in the ER by fusing it to the N-terminus of HDEL.

We then made several mutations in the CXXC motif of Trx (74). Three of these mutations were specific changes. CGPS or SGPC Trx cannot form a disulfide bond within their active sites, and thereby mimic the shufflease mutant of PDI and its regioisomer. CGHC Trx has a reduction potential of $E^{\circ\prime} = -0.235$ V, which approaches that of PDI (76, 77). To generate enzymes with an even wider range of chemical properties, the codons for Gly33 and Pro34 were mutated in tandem to codons for all 20 amino acid residues. The resulting pool codes for 400 double-mutants of Trx. cDNA's coding for the individual mutant Trx's and the pool of random Trx's were tested for their abilities to complement *pdi1*Δ *S. cerevisiae*.

In analogy to PDI, CGPS, but not SGPC, Trx can replace PDI (74). This result indicates that Cys32 but not Cys35 is an essential residue in catalysis of native disulfide bond formation. CGHC Trx, but not the wild-type enzyme, can substitute for PDI. This result points to reduction potential as a key determinant in the efficient catalysis of disulfide bond formation in vivo. cDNA's for two proteins from the random pool were also able to complement *pdi1*Δ yeast. Surprisingly, the sequences of the corresponding plasmids indicated that each protein had a tryptophan residue in either position 33 or 34. Complementation of *pdi1*Δ yeast with cDNA's encoding Trx demonstrates that any roles ascribed to PDI, other than its catalysis of the formation of native disulfide bonds, are not essential to eukaryotic cells. In other words, PDI is first and foremost a catalyst of the activity for which it was named and needs only its Trx domains for this activity.

VII. CORRELATING BIOLOGY AND CHEMISTRY OF THE CXXC MOTIF

We used Trx to correlate the biological and chemical properties of proteins bearing a CXXC motif (74). Complemented cells containing different CXXC motifs grow at different rates. Thus, an intrinsic property of the CXXC motif must limit cell viability. What is this property?

The reduction potential of the CXXC motif provides a measure of the relative stability of its dithiol and disulfide forms. CGHC, CWGC, and CVWC Trx

have reduction potentials that are significantly higher than that of wild-type Trx (74). The increase in reduction potential indicates that the mutations stabilize the reduced form of Trx, relative to the oxidized form.

The thiol pK_a of Cys32 (underlined) in the CXXC motif provides a measure of the relative stability of its protonated and unprotonated forms. The values of pK_a for the two mutants of Trx were depressed relative to that of wild-type Trx (74). This decrease indicates that the mutations at positions 33 and 34 have stabilized the thiolate form of Cys32.

The increased reduction potentials and decreased pK_a values of the Trx mutants are themselves correlated. Thiols of low pK_a have been shown to form less stable disulfide bonds in both organic (78) and enzymic (79) systems.* The likely physical basis for the anomalously low pK_a of Cys32 in wild-type Trx is its position at the N terminus of an α helix (80). There the positive dipole of the helix appears to stabilize the negative thiolate form of Cys32 (81,82). Molecular modeling of the Trx mutants suggests that replacing Pro34 removes conformational constraints, allowing the thiol of Cys32 to interact even more strongly with the N terminus of the helix (74).

VIII. ESSENTIAL PROPERTIES OF THE CXXC MOTIF

Two biophysical properties of the CXXC motif are apparently critical to cell viability (Fig. 6). An increase in the reduction potential of a CXXC motif increases the fraction of the enzyme that is present in the reduced form. The higher reduction potential observed in complementing enzymes is consistent with the necessity of having an enzymic thiol or thiolate in the ER. The ability of CGPS Trx but not wild-type Trx to confer viability to *pdi1Δ S. cerevisiae* also supports this conclusion. Reduction potential is linked to another important equilibrium—a thiol must be deprotonated to act as a nucleophile in a thiol–disulfide interchange reaction. Mutation of the noncysteine residues in the CXXC motif can lower the pK_a of the nucleophilic thiol, thereby increasing at any pH the fraction of the enzyme that exists in the thiolate form. Because the thiol pK_a for wild-type Trx is already below the ambient pH of the yeast ER, cell viability is less sensitive to changes in pK_a than to changes in reduction potential.

Catalysis is a cyclic process (83). A redox-active catalyst must cycle between reduced and oxidized forms. A balance must be achieved between the stability of the dithiol and disulfide forms for both substrate turnover and catalyst regeneration to occur efficiently. For example, a CXXC motif with a high reduction potential [such as DsbA, with $E^{\circ\prime} = -0.89$ (84)] would not necessarily be a good catalyst of disulfide bond formation in the ER because it exists almost exclusively in the dithiol form rather than in the disulfide form that initiates disulfide bond formation in a substrate. The reduction potential of PDI matches that of the ER (Fig. 5). Thus, wild-type PDI and the mutants of Trx that complement *pdi1Δ* cells exist in the ER as a near-equimolar mixture of reduced and oxidized forms, allowing substrate turnover and catalyst regeneration to proceed with comparable facility. In contrast, wild-type Trx and DsbA have reduction potentials that are too extreme for significant recycling to occur in the ER. Interestingly, the recycling of these two enzymes in vivo is coupled to other enzyme-catalyzed redox reactions (62,85).

*An equation that relates changes in the reduction potential of a CXXC motif to changes in the pK_a of its N-terminal thiol can be derived from eq. 38 of Ref. 78. At pH 7.0 and 30°C:

$$\Delta E^{\circ\prime}(\mathrm{V}) = -0.036\,\Delta \mathrm{p}K_a$$

This empirical relationship is in agreement with the extensive data for DsbA mutants in Ref. 79.

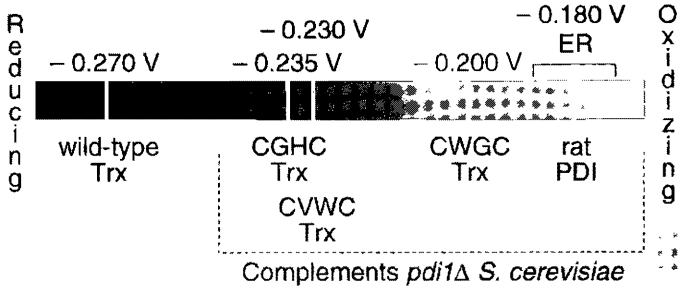

Fig. 5 Spectrum of reduction potentials for CXXC motifs that have been tested for complementation of *pdi1Δ S. cerevisiae* (74).

The effects of pK_a on catalysis are more complex (3). An isomerization reaction (Fig. 3) begins with the nucleophilic attack of a thiolate on a disulfide bond. Thiols of low pK_a are ionized more often (giving higher reaction rates) but are intrinsically less nucleophilic (78). These effects counteract such that rate constants for thiol–disulfide exchange reactions are maximal when the pK_a of the thiol equals the pH of the solution (3). The pK_a of a thiol also affects its electrophilicity, which is necessary for regenerating the catalyst. Thiols of low pK_a are better leaving groups from disulfide bonds (78). A compromise must therefore be achieved to maximize catalytic efficiency. A typical cysteine residue has a side chain pK_a of 8.7. The CXXC motifs that complement *pdi1Δ* yeast have thiol pK_a values of 6–7. Apparently, the catalysis of native disulfide bond formation has resulted in a lower thiol pK_a so as to maximize its ionization and electrophilicity without minimizing its nucleophilicity.

Nature often resorts to compromise. For example, enzymes have evolved to function under a variety of constraints (86,87). Because cells containing different CXXC motifs grow at different rates, an attribute of the CXXC motif must be of great consequence. The correlation shown in Fig. 6 indicates that this attribute is the fraction of the CXXC motif that is in the thiolate form. This

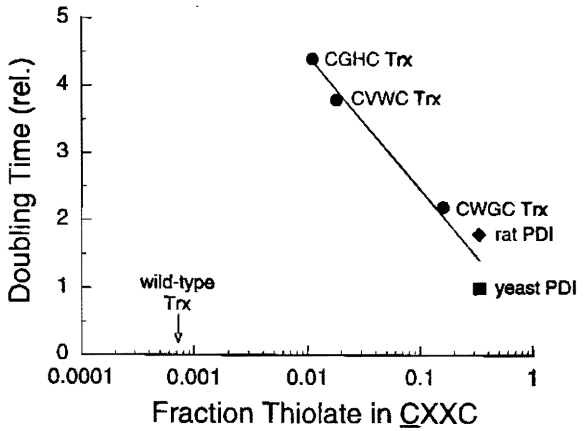

Fig. 6 In vivo (ordinate) and in vitro (abscissa) properties of CXXC motifs. The fraction thiolate is calculated from the measured reduction potentials and thiol pK_a values of the CXXC motifs with the Nernst and Henderson–Hasselbalch equations (74). Values are for pH 7.0 and $E^{o'}$ = -0.180 V, as found in the ER (4). The rate of growth of *pdi1*Δ *S. cerevisiae* cells complemented with a plasmid that directs the expression of a protein bearing the CXXC motif.

correlation is extended in Fig. 7, which shows how the fraction thiolate in the ER varies with both CXXC reduction potential and thiol pK_a. The gray surface in Fig. 7 denotes combinations of reduction potential and pK_a that would yield fraction thiolates less than that of CGHC Trx in the ER. To date, no enzyme with parameters in this gray area has been shown to replace PDI in vivo.

IX. CXXC VS. CXXS

Replacing a cysteine residue in a CXXC motif with a serine alters a fundamental property—the ability to form a disulfide bond within the motif. Redox catalysis by the CXXC motif occurs by a ping-pong mechanism. First, a disulfide bond is lost in the CXXC motif as one is gained in a substrate (such as a reduced protein). To complete the catalytic cycle, a disulfide bond is lost in a second substrate (such as oxidized glutathione) as one is gained in the CXXC motif. Enzymes with CXXC motifs thus interact with two substrates in two distinct steps. In contrast, because CGPS and SGPC Trx cannot form a disulfide bond in their CXXC motifs, these enzymes cannot catalyze a redox reaction except in a single step that involves two substrates. Apparently, this constraint makes these enzymes inefficient in vitro catalysts of redox reactions (54,58,88).

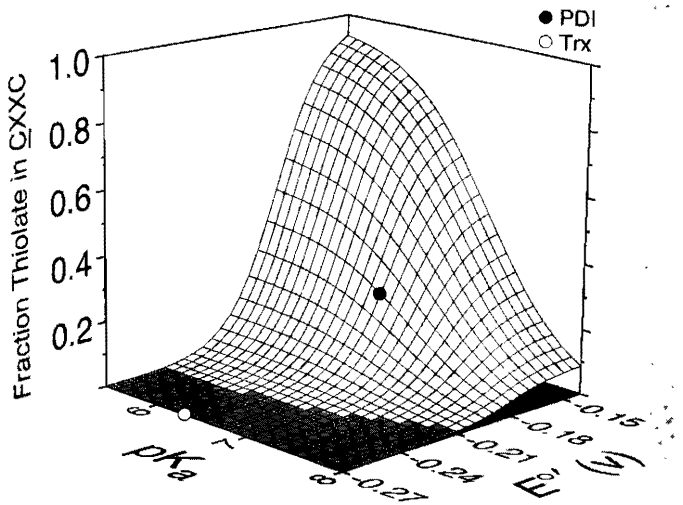

Fig. 7 Calculated relationship between the pK_a and reduction potential of a CXXC motif and the fraction that will be present as a thiolate in the ER of a eukaryotic cell. Surface is drawn for an ER with pH 7.0 and $E^{o'}$ = –0.180 V (4). The values of PDI and Trx are indicated. The gray surface designates combinations of pK_a and $E^{o'}$ that yield a fraction thiolate <0.01. No CXXC motif having a fraction thiolate in the gray area has been shown to complement *pdi1*Δ *S. cerevisiae*.

If a CXXS sequence can replace the CXXC motif, why does PDI have a CXXC motif? One possibility is that a CXXS sequence may become trapped as a mixed disulfide with a substrate. Such a trap could arise if the enzyme–substrate disulfide bond is inaccessible to both substrate thiols and cellular glutathione. Then, the mixed disulfide could remain intact and thereby waste cellular resources. In contrast, the EM of the thiols in the active site of PDI is 2 mM, which always enables it to escape from a mixed disulfide by forming a disulfide bond within the CXXC motif.

Alternatively, a CXXS sequence may be susceptible to inactivation by adventitious oxidation by molecular oxygen. For example, a sulfenic acid [RS-OH ⇌ RS(O)H] formed in a CXXS sequence could be oxidized further to a sulfinic acid [RS(O)-OH ⇌ $RS(O)_2H$] and ultimately to a sulfonic acid [$RS(O)_2$-OH]. In contrast, a sulfenic acid formed in a CXXC motif could be rescued by formation of a half-cystine. Thus, a CXXS sequence could be more useful to cells grown under less oxidizing conditions. Further, having both a CXXC motif and a CXXS motif from endogenous PDI and Eug1p, respectively, could provide cells with a selective advantage.

Much evidence suggests that dithiol oxidation is random during the early stages of protein folding (89,90). Classic studies on the oxidative folding of reduced bovine protease trypsin inhibitor indicate that nonnative intermediates accumulate during the folding process (1). In contrast, recent work suggests that the well-populated intermediates contain only native disulfide bonds (2). Still, to reach the final conformation, these intermediates must rearrange by forming species with nonnative disulfide bonds. PDI has been shown to catalyze this process by rescuing kinetically trapped intermediates (24). The isomerization activity of PDI may therefore be required both in normal protein folding pathways (24) and in the rescue of proteins that have become misfolded or aggregated (91,92). Thus, as proposed by Anfinsen (13) more than 30 years ago, the essential function of PDI is to isomerize nonnative disulfide bonds—to be a shufflease.

X. ENVOI

Complementation of *PDI1* null mutants of *S. cerevisiae* can be used to reveal the imperatives for the formation of native disulfide bonds in cellular proteins. Genetic and biophysical data demonstrate that the primary role of PDI is catalysis by its Trx domains-other functions ascribed to PDI (such as chaperone and antichaperone activity) are less relevant. The critical attribute of a CXXC motif like that in PDI is its ability to provide a thiolate in the cell. Although a CXXS sequence can substitute for CXXC, the second cysteine residue may allow for the intramolecular reversal of deleterious side reactions. Finally, nonnative disulfide bonds do exist in vivo, and the role of the CXXC (or CXXS) motif is to unscramble these bonds.

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