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The CXXC motif: imperatives for the formation of native disulfide bonds in the cell

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The rapid formation of native disulfide bonds in cellular proteins is necessary for the efficient use of cellular resources. This process is catalyzed *in vitro* by protein disulfide isomerase (PDI), with the *PDI1* gene being essential for the viability of *Saccharomyces cerevisiae*. PDI is a member of the thioredoxin (Trx) family of proteins, which have the active-site motif CXXC. PDI contains two Trx domains as well as two domains unrelated to the Trx family. We find that the gene encoding *Escherichia coli* Trx is unable to complement *PDI1* null mutants of *S.cerevisiae*. Yet, Trx can replace PDI if it is mutated to have a CXXC motif with a disulfide bond of high reduction potential and a thiol group of low pK_a. Thus, an enzymic thiolate is both necessary and sufficient for the formation of native disulfide bonds in the cell.

Keywords: chaperone/disulfide bond/protein disulfide isomerase/protein folding/thioredoxin

Introduction

Misfolded proteins are costly to a cell. Non-functional, they have consumed valuable cellular resources in their synthesis. Moreover, their accumulation can limit cell growth by saturating secretory and other pathways (Robinson and Wittrup, 1995).

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 (Creighton, 1977; Weissman and Kim, 1991). This complexity arises because the thiol groups and disulfide bonds of proteins can undergo three distinct chemical reactions: dithiol oxidation, disulfide reduction and disulfide isomerization (Gilbert, 1990). Of the three, only disulfide isomerization is a redox-independent process (Figure 1).

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 (Gilbert, 1990). Accordingly, disulfide bonds are rare in the cytosol, where the reduction potential is low [$E^{\circ\prime}$ = –0.230 V (Hwang *et al.*, 1992)]. In contrast, disulfide bonds are common in the endoplasmic reticulum (ER), where the reduction potential is high ($E^{\circ\prime}$ = –0.172 to –0.188 V). The effective molarity of two thiols in a typical unfolded protein has been estimated to be 50 mM relative to two molecules of glutathione (Lin and Kim, 1989, 1991), which corresponds to $E^{\circ\prime}$ = –0.22 V (Chivers *et al.*, 1996). Thus, transit from the cytosol to the ER dramatically increases the tendency of protein thiols to form disulfide bonds, both native and non-native.

Eukaryotic cells contain an ensemble of proteins that orchestrate the proper folding of other proteins (Freedman, 1992). Of these proteins, only protein disulfide isomerase (PDI) is known to catalyze the formation of native disulfide bonds. PDI, a 57 kDa resident of the ER, was first isolated 30 years ago by Anfinsen and contemporaries (Goldberger *et al.*, 1963; Venetianer and Straub, 1963), who favored 'the hypothesis that the enzyme is a general and nonspecific catalyst for disulfide interchange in proteins containing disulfide bonds' (Givol *et al.*, 1964). These early studies demonstrated that PDI can act as a catalyst for the isomerization of disulfide bonds *in vitro*.

In contrast, LaMantia and Lennarz (1993) claimed that PDI is not needed for the catalysis of disulfide bond isomerization in *Saccharomyces cerevisiae*. This claim has spawned the notion that PDI acts instead as a chaperone or 'anti-chaperone' (Noiva *et al.*, 1993; Puig and Gilbert, 1994; Puig *et al.*, 1994; Wang and Tsou, 1993). The data of LaMantia and Lennarz (1993) only show, however, that PDI is not essential for the formation of disulfide bonds. Their results do not address the role of PDI in the catalysis of disulfide bond isomerization (Figure 1). Recent results from our laboratory indicate that catalysis of isomerization is indeed the essential function of PDI in the cell (Laboissière *et al.*, 1995b).

PDI is homologous to thioredoxin (Trx), a 12 kDa cytosolic reducing agent for ribonucleotide reductase and other proteins (Holmgren, 1985; Buchanan *et al.*, 1994). PDI contains two Trx domains as well as two other domains unrelated to Trx (Edman *et al.*, 1985). Overall, the proteins share ~30% amino acid identity and have similar active sites: Cys-Gly-His-Cys in each Trx domain of PDI (Edman *et al.*, 1985) and Cys-Gly-Pro-Cys in Trx (Höög *et al.*, 1985; Lim *et al.*, 1985). Unlike PDI from any organism, *Escherichia coli* Trx is a small, well-characterized protein. A crystalline structure of the oxidized form is known to 1.68 Å resolution (Katti *et al.*, 1990), as are solution structures of both the oxidized and the reduced forms (Figure 2) (Jeng *et al.*, 1994). Although *PDI1* is essential for the viability of *S.cerevisiae* (Scherens *et al.*, 1991), *trxA* is not essential for *E.coli* (Holmgren, 1976).

We have used *E.coli* Trx to reveal which domains of PDI are essential *in vivo* and the properties of the Cys-Xaa-Xaa-Cys (hereafter, CXXC) motif that are most important to eukaryotic cells. Specifically, we have assessed the viability of *pdi1Δ S.cerevisiae* cells containing CGPC (i.e. wild-type) or CGHC (which has a PDI-like active site) Trx in their ER. We did the same with cells

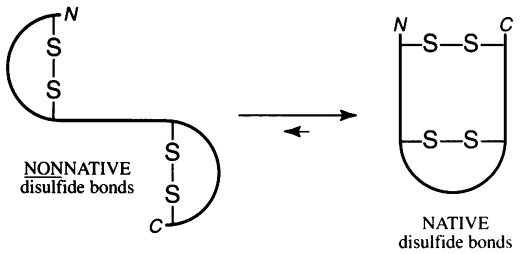

Fig. 1. Disulfide bond isomerization reaction catalyzed by PDI and other proteins with a CXXC (or CXXS) motif.

containing Trx mutants in which the two residues between the active-site cysteine residues had been made random. Finally, we tested the viability of cells containing CGPS or SGPC Trx, two enzymes that cannot form a disulfide bond within their active sites. Our results reveal that the Trx domains of PDI are essential to *S.cerevisiae*, and that an enzymic thiolate is both necessary and sufficient for the catalysis of native disulfide bond formation in cellular proteins.

Results

Production of Trx in S.cerevisiae

To illuminate the function of the CXXC motif in eukaryotic cells, we designed a way to target and retain *E.coli* Trx in the ER of *S.cerevisiae*. Previously, we had used plasmid YEpWL to direct the expression of rat PDI (Laboissière *et al.*, 1995a,b) and bovine pancreatic RNase A (delCardayré *et al.*, 1995) in *S.cerevisiae*. Here, we constructed a plasmid, YEpWL.TRX, that directs the expression of Trx fused to the C-terminus of the α-factor pre–pro segment (Brake *et al.*, 1984) and the N-terminus of HDEL (Pelham *et al.*, 1988). This plasmid can replicate in both *E.coli* and *S.cerevisiae*, and contains the selectable *TRP1* and *LEU2*-d genes. The expression of the *trxA* gene in YEpWL.TRX is controlled by the glucose-regulated ADH2–GAPDH promoter. We used oligonucleotide-mediated site-directed mutagenesis to change the codons for the CGPC active site of wild-type Trx to those for CGHC, CGPS and SGPC (Table I). Molecular modeling and structural studies suggest that these mutants are likely to adopt a structure similar to that of wild-type Trx (Eklund *et al.*, 1991; Krause *et al.*, 1991; Dyson *et al.*, 1994).

Our goal in replacing Pro34 with a histidine residue was to make the reduction potential of the CXXC motif of Trx more like that of PDI (Krause *et al.*, 1991; Lundström *et al.*, 1992). To generate enzymes with a still wider range of reduction potentials, we mutated the codons for Gly33 and Pro34 in tandem to codons for all 20 amino acid residues. The resulting pool codes for 400 double mutants of Trx. Codons 33 and 34 were considered to be random in this pool because G, A, T and C were found in positions one and two, and G, C and T were found in position three of each codon (data not shown). A plasmid library was obtained from ~200 bacterial transformants.

Our goal in replacing Cys32 or Cys35 with a serine residue was to alter a fundamental property of Trx: its ability to form a disulfide bond within its active site. Redox catalysis by the CXXC motif occurs in two steps. First, a disulfide bond is lost in a substrate (such as an oxidized protein) as one is gained in the CXXC motif. To

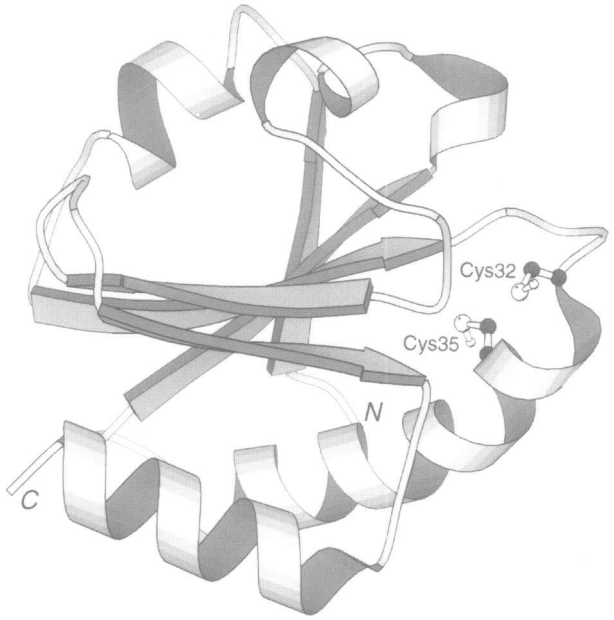

Fig. 2. Three-dimensional structure of the reduced form of *E.coli* thioredoxin (Dyson *et al.*, 1990). The only side chains shown are those of the cysteine residues of the CXXC motif. The figure was created with the program MOLSCRIPT v1.2 (Kraulis, 1991).

complete the catalytic cycle, a disulfide bond is formed in a second substrate (such as two molecules of glutathione) as one is lost in the CXXC motif. Enzymes with CXXC motifs thus interact with two substrates in two distinct steps. In contrast, since 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 catalysts of redox reactions (LaMantia and Lennarz, 1993; Laboissière *et al.*, 1995b; Wunderlich *et al.*, 1995).

Complementation of pdi1Δ S.cerevisiae

Plasmids that code for CGHC and CGPS Trx, but not wild-type or SGPC Trx, can complement *pdi1*Δ yeast (Table I). The ability of a plasmid to complement was determined by plasmid shuffling. Here, trp^+ ura^+ cells, which contain plasmids pCT37 (*S.cerevisiae PDI1* and *URA3*) and YEpWL.TRX (*TRX* and *TRP1*), were grown on solid medium containing 5-fluoro-orotic acid (5-FOA) to select against pCT37. Colonies of *pdi1*Δ *S.cerevisiae* complemented with pRS424 (*S.cerevisiae PDI1*) were visible after 2–3 days. In contrast, colonies of cells complemented with a mutated YEpWL.TRX appeared in 6–8 days. After this time, the appearance of colonies of cells transformed with YEpWL (no insert) precluded differentiating between true and false positives by their growth alone. True complementing cells were trp^+ ura^-. Immunoblot analysis confirmed the presence of Trx in the microsomal fraction from *pdi1*Δ yeast cells after plasmid shuffling with YEpWL. TRX, but not pRS424 (data not shown).

Complementation by a plasmid that codes for CGHC Trx, but not by one that codes for wild-type Trx, points to reduction potential as a key determinant in the efficient catalysis of disulfide bond formation *in vivo*. Complementation by a plasmid that codes for CGPS, but not by

Table I. *In vivo* and *in vitro* properties of PDI and Trx

Protein	Nucleotide sequence of CXXC[a]	Doubling time of complemented *pdi1*Δ yeast (rel.)	$E^{\circ'}$ of CXXC	pK_a of CXXC	Thiolate form of CXXC in ER (%)[b]
Yeast PDI	TGTGG(CT)CACTGT	1.0	n.d.	n.d.	n.d.
Rat PDI	TGTGG(CT)CACTGC	1.8 ± 0.2[c]	–0.180[d]	6.7[e]	33
Wild-type Trx	TGCGGTCCGTGC	n.c.	–0.270[f]	6.28	0.075
CGHC Trx	TGCGGTCACTGC	4.4 ± 0.8	–0.235[g]	n.d.	1.1[h]
CVWC Trx	TGCGTGTGGTGC	3.8 ± 0.4	–0.230	5.86	1.8
CWGC Trx	TGCTGGGGTTGC	2.2 ± 0.2	–0.200	5.94	16
CGPS Trx	TGCGGTCCGTCT	4.3 ± 0.5	–	n.d.	83[h]
SGPC Trx	TCTGGTCCGTGC	n.c.	–	–	–

n.d., not determined; n.c., no complementation.
[a]PDI has two CXXC motifs; underlined sequences were created in this work.
[b]Calculated for the thiol of the N-terminal cysteine residue of the CXXC motif by using the Nernst and Henderson–Hasslebalch equations at pH 7.0 with $E^{\circ'}$ = –0.180 V (Hwang *et al.*, 1992).
[c]Laboissière *et al.* (1995).
[d]Lundström and Holmgren (1993).
[e]Hawkins and Freedman (1991).
[f]Moore *et al.* (1964).
[g]Krause *et al.* (1991).
[h]Assuming that the pK_a of CXXC is unchanged by the mutation.

one that codes for SGPC Trx, indicates that Cys32 but not Cys35 is an essential residue in the catalysis of native disulfide bond formation. Two plasmids from the random pool are also able to complement *pdi1*Δ yeast (Table I). These plasmids coded for a protein that has a tryptophan residue in either position 33 or 34. (Recently, we found that CWDC Trx can also replace PDI in yeast.) Because only a single codon codes for Trp, its appearance in the genetic selection attests to the extensive scope of mutants in the random pool. The inability of our selection to uncover a plasmid encoding CGHC Trx is not unexpected because we did not survey the entire CXXC pool.

Function of Trx in vivo

The ability of Trx to function *in vivo* was assessed by the growth rate of complemented *pdi1*Δ *S.cerevisiae* cells. The doubling time of *pdi1*Δ *S.cerevisiae* cells complemented with yeast *PDI1* is 3.0 h. Complemented cells containing CWGC Trx double 2.2-fold more slowly than those containing yeast PDI (Table I). Cells containing CVWC, CGPS or CGHC Trx double 3.8- to 4.4-fold more slowly than those containing yeast PDI.

Purification of Trx from S.cerevisiae

The purification of Trx from *S.cerevisiae* was made facile in two ways. First, experiments were designed so that the same plasmid could be used for both complementation analysis (in *pdi1*Δ strain YPH274) and protein production (in vacuolar protease-deficient strain BJ2168). Second, much of the Trx produced is secreted into the growth medium—away from intracellular proteins. Trx produced in this system has the same mobility during SDS–PAGE as does Trx isolated from *E.coli*, indicating that the α-factor pre–pro segment is removed. [For a detailed discussion of this expression system, see Laboissière *et al.* (1995a).]

Pure protein was obtained from the medium by precipitation with phosphoric acid, gel-filtration chromatography and anion-exchange chromatography. After gel-filtration chromatography, the fractions containing Trx also contain a high concentration of inorganic phosphate. Accordingly, Trx elutes in the flow-through from the anion-exchange column. Reloading of this flow-through and application of an NaCl gradient yields 2 mg of pure Trx per liter of *S.cerevisiae* culture. This yield is adequate for the biophysical characterization of mutant Trxs.

Reduction potential of the CXXC motif

The reduction potential of the CXXC motif provides a measure of the relative stability of its dithiol and disulfide forms. The interconversion between these two forms is catalyzed by thioredoxin reductase (TR), with the concomitant interconversion of $NADP^+$ and NADPH. Thus, in the presence of TR, the characteristic UV absorbance of NADPH reports on the relative stability of the dithiol and disulfide forms of Trx.

We used the TR-coupled assay to determine the reduction potential of the active-site disulfide bonds of the CXXC motifs. CGHC, CWGC and CVWC Trx are substrates for TR, and each has a reduction potential that is higher than that of wild-type Trx (Table I). The measured reduction potential of CWGC Trx is actually a lower limit. Even at low initial concentrations of NADPH and CWGC Trx, the addition of a high concentration (5 mM) of $NADP^+$ oxidizes only a small fraction of CWGC Trx. The increase in reduction potential indicates that the mutations have stabilized the reduced form of Trx.

pK_a of thiol in the CXXC motif

The pK_a of the Cys32 (underlined) thiol in the CXXC motif provides a measure of the relative stability of its protonated and unprotonated forms. The fluorescence intensity of the indole side chain of Trp28 in reduced Trx changes with pH. This change had been proposed to arise from the titration of the thiol in the side chain of Cys32 (Holmgren, 1972; Reutimann *et al.*, 1981), and the pK_a of 6.35 first assigned to Cys32 by fluorescence titration was later confirmed by NMR spectroscopy (Forman-Kay *et al.*, 1992).

We used the change in fluorescence intensity to determine the pK_a of Cys32 in Trx. For wild-type Trx, we obtained a pK_a of 6.28 (Table I), which does not differ

significantly from values reported previously. The pK_a values of the two mutants of Trx were depressed by 0.4–0.5 pH units relative to that of wild-type Trx. This decrease indicates that the mutations at positions 33 and 34 have stabilized the thiolate form of Cys32.

Recently, Jeng *et al.* (1994) asserted that the pK_a monitored by fluorescence titration of Trx is not that of Cys32. Rather, they suggested that titration of His6 gives rise to the observed pK_a. Accordingly, we also used two-dimensional ^1H NMR spectroscopy as an independent method to determine the pK_a of Cys32 in our mutant enzymes (P.T.Chivers, K.E.Prehoda and R.T.Raines, unpublished results). Preliminary NMR data indicate that the pK_a values of Cys32 in the mutant enzymes are ~0.5 units lower than that of Cys32 in wild-type Trx (Dyson *et al.*, 1991; Jeng *et al.*, 1995). In contrast, the pK_a of His6 in the CWGC enzyme remains unchanged and that in the CVWC enzyme actually increases. Thus, fluorescence and NMR titrations yield consistent results: Cys32 in a mutant Trx that can replace PDI has a lower pK_a than does Cys32 in wild-type Trx. Finally, it is worth noting that discrepancies between thiol pK_a values determined by NMR and fluorescence spectroscopy may result from the low deuterium fractionation factor of sulfhydryl groups (Schowen, 1977). NMR titrations performed in deuterated water must be corrected for this anomaly.

Discussion

In all species, the dominant role of Trx is as a cytosolic reducing agent (Holmgren, 1985; Buchanan *et al.*, 1994). Of small size, Trx does not possess the multiplicity of non-redox functions exhibited by PDI (Pihlajaniemi *et al.*, 1987; Wetterau *et al.*, 1990). For example, Trx does not bind a tripeptide that was used to identify a peptide-binding region in PDI (Noiva *et al.*, 1993). Nevertheless, the amino acid residues that surround the CXXC motifs of PDI and Trx are those that are most conserved between the two proteins (Edman *et al.*, 1985).

The presence of Trx in all types of organisms (Eklund *et al.*, 1991) 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. ERp72, a protein of the eukaryotic ER, has three CXXC motifs and is able to complement *pdi1*Δ yeast (Günther *et al.*, 1993). 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 (Bardwell *et al.*, 1991). DsbA shares no sequence similarity with *E.coli* Trx apart from the active site, but the two proteins do have similar three-dimensional structures (Martin *et al.*, 1993). The widespread presence of the CXXC motif points to a critical role in cellular function (Chivers *et al.*, 1996).

By complementing *pdi1*Δ yeast with Trx, we have demonstrated that any roles ascribed to PDI, other than its catalysis of the formation of native disulfide bonds, are not essential. 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. In addition, the ability of a cytosolic protein from a prokaryotic cell to function in the ER of a eukaryotic cell heralds the versatility of the CXXC motif. Further, our genetic and biophysical analyses of the CXXC motif reveal the requirements for catalysis of protein disulfide bond isomerization in the eukaryotic cell.

Role of PDI in the cell

Catalysis of disulfide bond reduction or dithiol oxidation depends on the redox environment (Gilbert, 1990). In contrast, during catalysis of disulfide bond isomerization, the substrate does not undergo a net change in oxidation state (Figure 1). Thus neither a redox-active catalyst nor a redox buffer (e.g. a mixture of reduced and oxidized glutathione) is necessary for catalysis. 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 (Darby and Creighton, 1995). 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. The energetics of such an isomerization reaction would be driven by the search for the most stable conformation of the substrate protein.

A redox-inactive catalyst, CGPS Trx, restores the viability of *pdi1*Δ yeast. Thus, reduction or oxidation of the catalyst itself is not necessary for the cell to live. This result indicates either that catalysis of disulfide bond isomerization is the essential function of the CXXC motif, or that this motif is unnecessary altogether. The lack of complementation with a plasmid encoding the redox-inactive SGPC Trx mutant indicates that Cys32 (Figure 2) is a critical residue, and that the function of the CXXC motif is to catalyze the isomerization of existing protein disulfide bonds (Figure 1). *In vitro* evidence supports this view. SGHC PDI is an ineffective catalyst of disulfide bond reduction and isomerization, and dithiol oxidation (Laboissière *et al.*, 1995b). CGHS PDI can catalyze only disulfide bond isomerization. Also, wild-type PDI acts after oxidizing equivalents have been introduced into a reduced substrate (Haggren and Kolodrubetz, 1988; Lyles and Gilbert, 1991).

Interestingly, a protein with a CXXS sequence already exists in the ER of *S.cerevisiae*, and this protein is able to complement *pdi1*Δ *S.cerevisiae* (Tachibana and Stevens, 1992). Its gene, *EUG1*, is not normally expressed well and complements only weakly when expressed at high levels. We propose that a CXXS sequence is less efficacious because of its inability to rescue itself from potentially harmful side reactions. A CXXC motif would be less susceptible to such inactivation because it could escape by forming an intramolecular disulfide bond. For example, a CXXS sequence is prone to irreversible oxidation: a sulfenic acid [RS-OH $\rightleftarrows$ RS(O)H] formed in a CXXS sequence could be oxidized further to a sulfinic acid [RS(O)-OH $\rightleftarrows$ $RS(O)_2H$] and then to a sulfonic acid [$S(O)_2$-OH]. Such a cascade, which would be initiated by molecular oxygen in the ER, could account for the wide variation observed in the doubling times of cells containing CGPS Trx (Table I). In contrast, a sulfenic acid formed in a CXXC motif could be converted to a half-cystine. Preliminary evidence suggests that a CXXS sequence is indeed more useful to cells grown under less oxidizing conditions (P.T.Chivers, D.J.Bauhs,Jr and R.T.Raines, unpublished results).

Trx mutants that can replace PDI

The substitutions for Gly33 and Pro34 found in the complementing Trx mutants are quite drastic. The success of these particular substitutions does, however, have a physical basis. An increase in reduction potential means that the stability of the reduced form has increased relative to the oxidized form. Mutation of a proline to another amino acid residue increases the conformational entropy of a polypeptide chain. The additional entropy would favor the reduced form because the oxidized form would be more strained if the protein were more flexible (Gilbert, 1990). Direct support for this argument comes from the reduction potentials of CGHC Trx (Krause *et al.*, 1991) and CGSC Trx (Lin and Kim, 1991), which are higher than that of wild-type Trx.

The appearance of a tryptophan, the most rare residue, in the complementing CXXC motifs may also be explicable. Tryptophan has the largest side chain of all natural amino acids. Its presence in the CXXC motif may serve to increase the reduction potential simply by providing a steric hindrance to disulfide bond formation. In addition, a hydrophobic residue in the CXXC motif could enhance the interaction of Trx with a misfolded protein. Analogously, a hydrophobic residue in T_4 glutaredoxin has been postulated to aid in the interaction of T_4 glutaredoxin with glutathione (Nikkola *et al.*, 1991).

The decreased pK_a values of the complementing CXXC motifs may be another manifestation of increased main chain flexibility. 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 (Figure 2) (Hol, 1985). There, the positive dipole of the helix can stabilize the negative thiolate form of Cys32 (Forman-Kay *et al.*, 1991). Molecular modeling of our Trx mutants suggests that replacing Pro34 allows the thiol of Cys32 to interact even more strongly with the N-terminus of the helix.

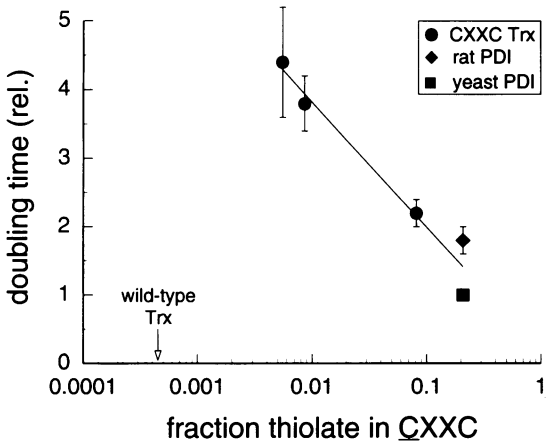

Fig. 3. *In vivo* (ordinate) and *in vitro* (abscissa) properties of CXXC motifs. Data are from Table I. A linear least-squares fit of the data gives: doubling time (rel.) = –2.0×log(fraction thiolate) + 0.51. This correlation and the doubling time (3.0 h) of *pdi1*Δ cells complemented with *PDI1* suggests that if wild-type Trx could replace PDI, then the doubling time of the resulting cells would be 20 h.

Requisite properties of a PDI

Two biophysical properties of the CXXC motif are apparently critical to cell viability (Table I; Figure 3). An increase in the reduction potential of a CXXC motif increases the fraction of the motif that is present in the reduced form. The higher reduction potential that we observed for Trx complementants 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 *pdi1*Δ *S.cerevisiae* also supports this conclusion. The reduction potential is linked to another important equilibrium: a thiol must be deprotonated to act as a nucleophile. We observed that the mutation of the non-cysteine residues in the CXXC motif can lower the pK_a of the nucleophilic thiol (Figure 3), thereby increasing at any pH the fraction of the enzyme that exists in the thiolate form. Nevertheless, the pK_a for wild-type Trx is already below the ambient pH of the yeast ER. Thus, in our system, cell viability is less sensitive to thiol pK_a than to CXXC reduction potential.

Catalysis is a cyclic process (Raines and Knowles, 1987). 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 that in DsbA; Figure 4) 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 (Figure 4). Thus, PDI (and our Trx mutants) 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 (Figure 4). *In vivo*, the recycling of the CXXC motifs in Trx and DsbA is coupled to other enzyme-catalyzed redox reactions (Holmgren, 1985; Bardwell *et al.*, 1993).

The effects of pK_a on catalysis are more complex (Gilbert, 1990). An isomerization reaction (Figure 1) begins with the nucleophilic attack of a thiolate on a

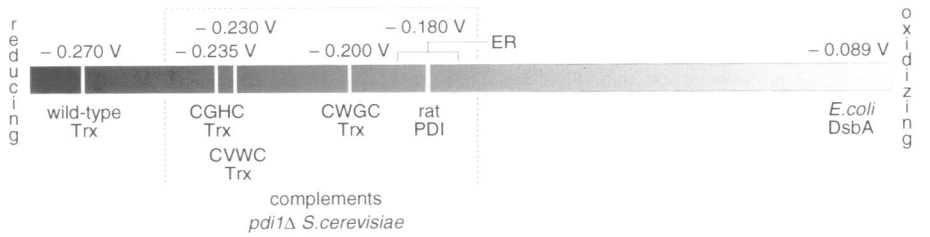

Fig. 4. Spectrum of the known reduction potentials for CXXC motifs. Trx mutants that can replace PDI have reduction potentials closer to that of the ER than does wild-type Trx. The reduction potential of DsbA is from Wunderlich and Glockshuber (1993).

disulfide bond. Thiols of low pK_a are ionized more often (giving higher reaction rates), but are intrinsically less nucleophilic. 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 (Gilbert, 1990), which is ~7.0 in the ER (Hwang *et al.*, 1992). The pK_a of a thiol also affects its electrophilicity, which is necessary for regenerating the catalyst. Thiols of low pK_a leave more rapidly from a disulfide. 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 *pdi1*Δ yeast have thiol pK_a values of 6–7 (Table I). Apparently, the need to catalyze native disulfide bond formation has resulted in a lower 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 (Benner, 1989; Burbaum *et al.*, 1989). Since cells containing different CXXC motifs grow at different rates (Table I), an attribute of the CXXC motif must be of great consequence. The correlation shown in Figure 3 indicates that this attribute is the fraction of the CXXC motif that is in the thiolate form.

Conclusions

We have used the complementation of *PDI1* null mutants of *S.cerevisiae* to reveal the imperatives for the formation of native disulfide bonds in cellular proteins. Our 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 anti-chaperone activity) are less relevant. Our analyses indicate that the critical attribute of a CXXC motif like that in PDI is its ability to provide a thiolate in the cell (Figure 3). Although a CXXS sequence can substitute for CXXC, we propose that the C-terminal cysteine residue is important because it provides an intramolecular anti-oxidant. Finally, our results and those of Laboissière *et al.* (1995b) suggest that non-native disulfide bonds do exist *in vivo* and that the role of the CXXC (or CXXS) motif is to unscramble these bonds (Figure 1; Chivers *et al.*, 1996).

Materials and methods

Plasmids, strains and reagents

Plasmid pTK10 was a generous gift of J.A.Fuchs (University of Minnesota). *Escherichia coli* TR was a generous gift of C.H.Williams,Jr (University of Michigan). Wild-type *E.coli* Trx was from Promega (Madison, WI). All enzymes for the manipulation of DNA were from Promega, except *Ava*II, which was from New England Biolabs (Beverly, MA).

Saccharomyces cerevisiae BJ2168 **a** (*prc1-407 prb1-1122 pep4-3 leu2 trp1 ura3-52*) was obtained from the Yeast Genetic Stock Center (Berkeley, CA). *Saccharomyces cerevisiae* strain YPH 274 α/**a** *pdi1*Δ::*HIS3* (*ura3-52 lys2-801 ade2-101 trp1-Δ1 his3-Δ200 leu2-Δ1*) was a generous gift from T.H.Stevens (University of Oregon). *Escherichia coli* strain DH11S *dut*$^-$*ung*$^-$ was a generous gift of D.Polayes (BRL, Gaithersburg, MD).

5-FOA was a generous gift of M.R.Culbertson (University of Wisconsin–Madison). Reagents for DNA synthesis were from Applied Biosystems (Foster City, CA), except for acetonitrile, which was from Baxter Healthcare (McGaw Park, IL). Bacto yeast extract, Bacto peptone and Bacto yeast nitrogen base without amino acids (YNB) were from Difco (Detroit, MI). Pepstatin A, phenylmethylsulfonylfluoride (PMSF), NADPH, $NADP^+$, 5,5-dithio-bis(2-nitrobenzoic acid) (DTNB), dithiothreitol (DTT) and 400–600 μm glass beads for yeast transformation were from Sigma (St Louis, MO). Ultrapure dideoxynucleotide triphosphates for mutagenesis were from Pharmacia (Piscataway, NJ). Concentrated phosphoric acid was from Mallinckrodt (Paris, KY). All other chemicals and reagents were of commercial or reagent grade, or better, and were used without further purification.

General methods

Manipulations of DNA, *E.coli* and *S.cerevisiae* were performed as described previously (Ausubel *et al.*, 1989; Shermann, 1991). DNA oligonucleotides were synthesized on an Applied Biosystems Model 392 DNA/RNA synthesizer using the β-cyanoethyl phophoramidite method (Sinha *et al.*, 1984), and were purified with Oligonucleotide Purification Cartridges from Applied Biosystems. DNA synthesis by the polymerase chain reaction (PCR) was performed with a DNA thermal cycler from Perkin Elmer Cetus (Norwalk, CT). DNA was purified with the Wizard MaxiPreps DNA Purification System (Promega), PCR Magic Preps (Promega) or GeneClean (Bio101, Vista, CA). Plasmids were transformed into *E.coli* cells with a GenePulser electroporator (Bio-Rad, Richmond, CA) and into *S.cerevisiae* cells as described previously (Elble, 1992). Dideoxynucleotide sequencing was performed with the Sequenase Version 2.0 kit from United States Biochemical (Cleveland, OH).

Yeast minimal medium (SD) contained (in 1 l) Bacto YNB (6.7 g), dextrose (2% w/v) and a supplemental nutrient mix (Ausubel *et al.*, 1989). Yeast rich medium (YEPD) contained (in 1 l) Bacto yeast extract (10 g), Bacto peptone (20 g) and dextrose (2% w/v). Variations of SD and YEPD media were also used. For example, YEP(1%)D medium contained 1% w/v dextrose. All media were prepared in distilled, deionized water and autoclaved before use.

Spectroscopy

The absorbance of UV and visible light was measured with a Cary Model 3 spectrophotometer equipped with a Cary temperature controller (Varian, Sugar Land, TX). The absorbance of cell cultures at 600 nm was recorded after diluting the cells such that A = 0.1–0.5. Fluorescence was recorded on an SLM 8000 fluorescence spectrometer from SLM Instruments (Urbana, IL) equipped with photon-counting electronics and data-collection software from ISS (Champaign, IL).

Expression and mutagenesis of trxA

The *trxA* gene was excised from pTK10 by digestion with *Xba*I and *Pst*I. The resulting fragment was purified and inserted into the corresponding sites in pBluescript II S/K, to yield plasmid pBST1. To signal for secretion, the α-factor pre–pro segment (Brake *et al.*, 1984) was added to the 5′ end of *trxA* by oligonucleotide-mediated site-directed mutagenesis (Kunkel, 1985) using oligonucleotide PC01 (5′ ACGCCAGGTTATGGTACCGCTGGATAAAAGAAGCGATAAAATT 3′) and single-stranded pBST1 prepared from *E.coli* strain DH11S. This mutation also created an *Acc*65I site for the in-frame insertion of *trxA* into yeast expression plasmid YEpWL.RNase A (delCardayré *et al.*, 1995).

To signal for ER retention (Pelham *et al.*, 1988), an HDEL sequence was added to the 3′ end of *trxA* by site-directed mutagenesis using oligonucleotide PC02 (5′ GCTAACCTGGCGCACGATGAGTTGTAA-CCGCGGTGCCCCGTCGCT 3′), to yield plasmid pBST7. This insertion displaced the stop codon by 12 nucleotides. The *Acc*65I–*Sal*I cassette from pBST7 was inserted into YEpWL.RNase A, to yield plasmid YEpWL.TRX. Single amino acid mutants of *trxA* were created in pBST7: P34H with oligonucleotide PC06 (5′ GGTGCGGTCACTGCAAAATG 3′), C35S with PC08 (5′ GCGGTCCGTCTAAAATGATC 3′) and C32S with PC21 (5′ GCAGAGTGGTCTGGTCCGTGCA 3′). After dideoxynucleotide sequencing, the mutated DNA fragments were subcloned into YEpWL.RNase A as described above.

Randomization of Gly33 and Pro34

The codons for Gly33 and Pro34 were mutated randomly by the PCR method of overlap extension (Ho *et al.*, 1989) using pBST7 as the template. The first round of mutagenesis used two separate reactions with oligonucleotides PC23 [5′ TGGGCAGAGTGGTGCNN(GCT)NN-(GCT)TGCAAAATGATC 3′] and PC05 (5′ AACGTGTTCAACCAA-GTCGACGGTATCGATAAGC 3′), or PC24 [5′ GGCGATCATTTTGC-A(GCA)NN(GCA)NNGCACCACTCTGC 3′] and PC01. The two fragments were purified, an aliquot of each was combined, and the resulting mixture was amplified by the PCR with oligonucleotides PC01 and PC05 to generate randomized *trxA*.

After fragment purification and restriction digestion, the *Acc*65I–*Sal*I cassette was inserted into YEpWL.RNase A that had been digested with

*Acc*65I and *Sal*I. The ligation product was transformed into *E.coli* TG1 by electroporation. After overnight growth at 37°C, each plate was washed with LB (2 ml) to suspend the colonies. The resulting suspension was diluted to 10 ml and grown for 6 h in LB containing extra ampicillin (300 μg/ml). An aliquot of this culture (1 ml) was used to inoculate LB (100 ml) containing ampicillin (100 μg/ml), and the resulting culture was grown overnight at 37°C. The plasmid library purified from this culture encoded Gly33All/Pro34All Trx.

Transformation of S.cerevisiae and plasmid shuffling

Plasmid shuffling refers to replacing a wild-type gene for a mutant gene in a haploid *S.cerevisiae* strain that has the wild-type gene on a plasmid, but not a chromosome (Sikorski and Boeke, 1991). Cells that undergo plasmid shuffling initially contain two different plasmids—wild type and mutant—each with a different selectable marker. By selecting against the plasmid containing the wild-type gene, mutant genes that confer viability (i.e. complement) can be identified. Here, we have selected against the *URA3* gene on plasmid pCT37, which directs the expression of *S.cerevisiae* PDI. The enzyme encoded by *URA3* produces a toxic catabolite from 5-FOA.

Transformed *S.cerevisiae* cells were plated on solid medium appropriate for plasmid selection. Wild-type and single-site mutants were transformed into haploid *pdi1*Δ YPH 274 containing pCT37. Twenty fresh transformants for each construct were picked and plated on an SD – trp master plate and a 5-FOA selection plate, both of which were incubated at 30°C. An aliquot of the plasmid library that codes for Gly33All/Pro34All Trx was also transformed into haploid *pdi1*Δ YPH 274 containing pCT37. Approximately 300 trp^+ transformants were plated on both SD – trp and 5-FOA media, which were then incubated at 30°C. Yeast *PDI1* on *TRP* plasmid pRS424 (M.C.A.Laboissière, S.L.Sturley and R.T.Raines, submitted) was used as a positive control, and YEpWL without an insert was used as a negative control. After growth on 5-FOA, colonies were replica plated on SD – trp and SD – ura media to confirm shuffling. Positive complementants were able to retain the trp^+ marker after several rounds of growth on solid YEPD medium, indicating the requirement of YEpWL.TRX in the absence of a selection for trp^+ (data not shown).

Yeast cell extracts

After plasmid shuffling, trp^+ ura^- YPH274 cells were grown at 30°C in YEP(1%)D medium (100 ml) until A = 0.7 at 600 nm. Cell extracts were then prepared as described previously (Bostian *et al.*, 1983). Pepstatin, EDTA and PMSF were added at standard concentrations to extraction buffers to inhibit protease activity (Deutscher, 1990).

Polyclonal antibodies to Trx

A chicken was injected with wild-type *E.coli* Trx (200 μg) and 7 days later boosted with additional Trx (100 μg). Eggs were collected 21 days after the initial injection and IgY isolated as described previously (Polson *et al.*, 1980).

Immunoblotting

Yeast cells were grown to late log phase in liquid medium (15 ml). Cell extracts were prepared as described above. Denaturing PAGE was carried out as described previously (Ausubel *et al.*, 1989). Protein samples were electroblotted onto nitrocellulose (Bio-Rad, Richmond, CA) with a Mini Trans-Blot apparatus from Bio-Rad. Chicken polyclonal antibodies against *E.coli* Trx (1:250 dilution), peroxidase-conjugated affinity-purified rabbit antibody to chicken IgG (Cappel Research Products, Durham, NC) and a Renaissance chemiluminescence detection kit (Dupont NEN, Boston, MA) were used to visualize protein according to the kit manufacturer's instructions.

Doubling time of S.cerevisiae

Haploid *pdi1*Δ cells complemented with *E.coli trxA* or yeast *PDI1* were grown in YEP(1%)D medium. These cultures were diluted with the same medium to a cell density of 3×10^6 cells/ml (A = 0.1 at 600 nm) and the resulting cultures (25 ml in a 125 ml culture flask) were grown at 30°C with shaking at 250 r.p.m. At 2 h intervals, an aliquot was removed and its A was measured at 600 nm. Log$A$ was plotted versus time, and the slope of the linear portion of the curve was determined by linear least-squares analysis. At least five different clones from each construct were analyzed. For each clone, the doubling time was calculated by dividing log 2 by the slope of the curve. For each construct, the mean and SD of the doubling times were calculated. Growth rates were normalized to the growth rate of *pdi1*Δ cells complemented with yeast *PDI1*.

Protein purification

Mutant and wild-type Trxs were produced in vacuolar protease-deficient *S.cerevisiae* BJ2168 using a protocol similar to that of Laboissière *et al.* (1995a). Briefly, to select for plasmid YEpWL.TRX, starter cultures (10 ml) were grown at 30°C in SD – trp medium. To produce protein, cultures (1.0 l) were grown at 30°C in YEP(1%)D medium with an initial A = 0.025 at 600 nm. Medium was collected when A = 6 (~24 h). Cells were removed by centrifugation at 4000 *g* for 10 min. The liquid medium was chilled by stirring in an ice–water bath for 30 min. The pH of the chilled medium was lowered to 3.5 by the addition of concentrated phosphoric acid. The precipitated protein was separated by centrifugation at 16 000 *g* for 45 min. The supernatant was discarded and the pellet resuspended in a minimal volume (<10 ml) of 100 mM potassium phosphate buffer (pH 8.0) containing EDTA (1 mM). The resulting solution was filtered through a 0.2 μm cellulose acetate filter (Nalgene, Rochester, NY).

The filtrate was applied (flow rate 1.5 ml/min) to a Pharmacia Hi Load 26/60 G-75 gel filtration column that had been equilibrated with sample resuspension buffer. Fractions containing Trx activity were pooled and loaded (flow rate 1 ml/min) on a Pharmacia MonoQ column that had been equilibrated with 20 mM imidazolium chloride buffer (pH 7.6). Trx eluted in the flow-through, and was reloaded on the MonoQ column and eluted with a linear gradient (12 + 12 ml) of NaCl (0.1–0.3 M) in 20 mM imidazolium chloride buffer (pH 7.6). Fractions containing Trx activity were pooled and concentrated with a 10 kDa cut-off Centriprep concentrator (Amicon, Beverly, MA).

Enzymatic activity

To aid protein purification, Trx activity was detected by using the coupled assay of Luthman and Holmgren (1982). In this assay, active Trx is detected by an increase in A at 412 nm due to the reduction of DTNB to produce 3-carboxylato-4-nitrothiophenolate (ε = 14 140/M/cm). The oxidized Trx formed during the reaction is reduced by TR and NADPH. Thioredoxin activity was assayed in a solution (0.80 ml) of 0.10 M Tris–HCl buffer (pH 8.0) containing EDTA (2 mM), NADPH (250 μM from a 20 mM stock solution in assay buffer) and DTNB (1.0 mM from a 100 mM stock solution in ethanol). The reaction was initiated by the addition of TR (2 μl of a 0.78 μg/μl solution).

Reduction potential

The reduction potential of Trx was determined by using the assay of Moore *et al.* (1964). In this assay, the reduction of Trx is accompanied by a decrease in A at 340 nm due to the TR-catalyzed reduction of NADPH (ε = 6200/M/cm). Here, TR (2 μl of a 0.78 μg/μl solution) was added to a solution (0.80 ml) of 0.10 M Tris–HCl buffer (pH 7.0) containing Trx (7–96 μg), NADPH (40–60 μM) and EDTA (2 mM). After equilibrium had been reached, $NADP^+$ (1.1 mM, final concentration) was added to generate a new equilibrium. Using the equilibrium concentrations of all species, $E^{\circ\prime}_{Trx}$ was determined using the equation:

$$E^{\circ\prime}_{Trx} = E^{\circ\prime}_{NADP+} + \frac{RT}{nF} \ln \frac{[Trx(SH)_2][NADP^+]}{[TrxS_2][NADPH]}$$

where $E^{\circ\prime}_{NADP+}$ = –0.315 V (Clark, 1960), R = 8.314 J/(K·mol), T = 298 K, n = 2 and F = 96 500 C/mol. At least three different starting concentrations of Trx were used to obtain a mean value of $E^{\circ\prime}$.

Thiol pK_a

The pK_a of Cys32 of Trx was determined by using fluorescence spectroscopy, as described by Holmgren (1972). Here, the fluorescence intensity (I) of reduced Trx (10 μg in 800 μl) was determined at pH 4.0–9.0 with buffers: sodium acetate, pH 4.0–5.5; potassium phosphate, pH 5.8–7.4; Tris–HCl, pH 8.0–9.0. Potassium chloride was added to each buffer to maintain an ionic strength of 0.10 M at each pH. The midpoint (pK_a) for the transition near neutral pH was calculated from a plot of $\log[(I_{max}-I)/(I-I_{min})]$ versus pH (Lee, 1970).

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