The Role of *ERO1* in Oxidative Protein Folding in the Endoplasmic Reticulum

by

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ABSTRACT

The formation of native disulfide bonds is critical for the folding and stability of many secreted proteins. We describe an essential *S. cerevisiae* gene, *ERO1*, which encodes a conserved ER membrane protein required for disulfide bond formation in the endoplasmic reticulum (ER). In a conditional *ero1-1* mutant, secretory proteins that would normally contain disulfide bonds, such as carboxypeptidase Y (CPY), are retained in the ER in reduced form, as shown by thiol modification with AMS. *ERO1* levels determine cellular oxidizing capacity, since mutation of *ERO1* causes hypersensitivity to the reductant DTT, whereas overexpression of *ERO1* confers resistance to DTT. Moreover, the thiol oxidant diamide can restore growth and secretion to *ero1* mutants. These results suggest that Ero1p provides the oxidizing equivalents utilized for disulfide bond formation in the ER.

Oxidizing equivalents are transferred directly from Ero1p to the abundant ER oxidoreductase PDI (protein disulfide isomerase) and its homolog Mpd2p. PDI is oxidized in wild-type cells, but reduced in the *ero1-1* mutant. Thiol-disulfide exchange between PDI and Ero1p is indicated by the capture of PDI-Ero1p mixed-disulfides. PDI oxidizes secretory proteins, since newly-synthesized CPY remains fully reduced in PDI-depleted cells. Mixed-disulfides between PDI and p1 CPY are also detected, indicating that PDI engages directly in thiol-disulfide exchange with this substrate. Together, these results define a pathway for protein disulfide bond formation in the ER wherein oxidizing equivalents flow from Ero1p to PDI (and Mpd2p) and then to substrate proteins through direct thiol-disulfide exchange reactions. Oxidized glutathione (GSSG) does not serve as an obligate intermediate in this pathway, since oxidative protein folding proceeds normally in a *gsh1Δ* mutant devoid of intracellular glutathione.

Mutational analysis of *ERO1* identifies two pairs of conserved, vicinal cysteines essential for Ero1p function. Mutation of Cys100, Cys105, Cys352, or Cys355 of Ero1p disrupts cell viability, CPY folding, and thiol-disulfide exchange between PDI and Ero1p. Cys100 of Ero1p may be preferentially attacked by PDI, while the Cys352-Cys355 disulfide may re-oxidize the Cys100-Cys105 cysteine pair. The properties of yeast Ero1p resemble those of E. coli DsbB.

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In loving memory of my grandfather, Sam Frand,
from whom I learned to value hard work and humor.

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Disulfide Bond Formation in Living Cells

OVERVIEW

The formation of native intramolecular disulfide bonds represents a critical step in the folding of many secreted proteins. Disulfide bonds can stabilize the final conformation of a folded protein, and intermolecular disulfide bonds can link the subunits of multi-protein complexes. Native disulfide bond formation involves the oxidation of protein thiols to form disulfide bonds as well as the rearrangement of non-native disulfides (Givol et al., 1964, Creighton, 1977, Weissman and Kim, 1991). Although the formation of structural protein disulfide bonds proceeds spontaneously in vitro, the process is error-prone and slow, taking hours to days (Anfinsen, 1973). In contrast, oxidative protein folding proceeds rapidly in vivo, where the process is catalyzed by thiol-disulfide oxidoreductases of the thioredoxin superfamily (Bergman and Keuhl, 1979, Bardwell et al., 1993, Goldberger et al., 1963).

The formation of structural protein disulfide bonds is compartmentalized in living cells, proceeding almost exclusively in the lumen of the endoplasmic reticulum (ER) in eukaryotic cells and in the periplasm of bacterial cells (Braakman et al., 1991, Derman and Beckwith, 1991). A pathway for protein disulfide bond formation has been described in the E. coli periplasm wherein the oxidoreductase DsbA donates disulfide bonds directly to substrate proteins (Bardwell et al., 1991). DsbA is then re-oxidized by DsbB, a second oxidoreductase residing in the cytoplasmic membrane (Bardwell et al. 1993, Missiakas et al., 1993). Electrons flow from DsbB to molecular oxygen or alternate acceptors via the electron transport chain (Bader et al., 1999, Kobayashi and Ito, 1999).

In eukaryotic cells, disulfide bond formation initiates upon translocation of newly-synthesized secretory proteins into the lumen of the ER, where oxidizing redox conditions support the net formation of protein disulfide bonds (Bergman and Keuhl, 1979, Braakman et al., 1991, Hwang et al., 1992). Glutathione (GSH) is the principal redox buffer in the secretory pathway, and oxidized glutathione (GSSG) has therefore long been thought to serve as the source of oxidizing equivalents for protein disulfide bond formation in vivo (Hwang et al., 1992, Bader et al., 1999b).

The abundant ER oxidoreductase Protein Disulfide Isomerase (PDI) is the principal catalyst for native disulfide bond formation in eukaryotic cells (Goldberger et al., 1963, Zapun et al., 1992). PDI is thought to function predominantly as an isomerase, but also as a catalyst for the GSSG-dependent oxidation of proteins thiols in the ER (Laboissière et al., 1995, Holst et al., 1997).

This thesis will describe a novel but conserved ER membrane protein, Ero1p, required for protein oxidation in the ER (Frand and Kaiser, 1998). Ero1p will be placed in a pathway for protein disulfide bond formation wherein oxidizing equivalents are transferred directly from Ero1p to PDI, enabling PDI to serve as a direct oxidant for secretory proteins (Frand and Kaiser, in press). As predicted by this model, GSSG is found to be dispensable for protein disulfide bond formation in vivo (Frand and Kaiser, 1998).

OXIDATIVE PROTEIN FOLDING IN THE ENDOPLASMIC RETICULUM

The environment within the ER lumen of eukaryotic cells is highly specialized to promote the oxidative folding of secretory proteins. Within this compartment, oxidizing redox conditions are sustained which thermodynamically and kinetically favor the formation of protein disulfide bonds (Hwang et al., 1992). The oxidized redox state of the ER is reflected by the relatively low ratio of the intraluminal GSH to GSSG concentration (Hwang et al., 1992). Catalysts for native disulfide bond formation, including PDI and other thioredoxin-like oxidoreductases, are also abundant in the ER, as are general chaperones, including Kar2p (Bip) (Zapun et al., 1992). In mammalian cells, disulfide bond formation initiates upon the translocation of nascent peptide chains into the ER lumen. Oxidative protein folding is completed post-translationally, as observed for secretory proteins including immunoglobulins, influenza hemagglutinin (HA), and the β-subunit of human chorionic gonadotropin (Bergman and Keuhl, 1979, Braakman et al., 1991, Segal et al., 1992, Huth et al., 1992). The folding, and thereby the intracellular transport, of proteins with native disulfide bonds can be selectively and

reversibly inhibited by the treatment of cells with reducing agents such as DTT (Braakman et al., 1992, Lodish and Kong, 1993, Jämsä et al., 1994). Analysis of the recovery of intracellular transport following the treatment of cells with DTT has revealed a requirement for energy and for the chaperone Kar2p (Bip) during oxidative refolding in vivo (Braakman et al., 1992b, Simmons et al., 1995).

THE ROLE OF INTRALUMINAL GLUTATHIONE

In eukaryotic cells, the redox state of the cytosol and the lumen of secretory organelles is reflected by the relative abundance of the reduced and oxidized forms of the essential tripeptide glutathione (L-γ-glutamyl-L-cysteinylglycine, GSH) (Hwang et al., 1992). In the cytosol, the ratio of the concentration of GSH to GSSG ranges from 30:1 to 100:1, with 1-10 mM total concentrations of glutathione (Hwang et al., 1992). In this reducing milieu, disulfide bond formation is generally restricted to the oxidation of catalytic cysteines in redox-active enzymes or regulatory cysteines in proteins sensing the intracellular redox state. One exception may be the formation of disulfide bonds in vaccina virus capsid proteins exposed to the cytosol of mammalian cells (Locker and Griffiths, 1999).

Cytosolic glutathione provides reducing equivalents for the reduction of protein disulfides, and also participates in the de-toxification of reactive oxygen species and xenobiotics (Meister, 1998). The redox state of glutathione in the cytosol is maintained by the NADPH-dependent reduction of GSSG, catalyzed by glutathione disulfide reductase (Muller, 1996). The enzyme γ-glutamylcysteine synthetase, encoded by the *GSH1* gene of yeast, catalyzes the first and rate limiting step in glutathione biosynthesis in the cytosol (Meister, 1998, Ohtake and Yabuchi, 1991). Consequently, mutation of *GSH1* results in the complete depletion of intracellular glutathione (Ohtake and Yabuuchi, 1991). Cells with a chromosomal deletion of *GSH1* are inviable on minimal medium, but can grow following the addition of sulfhydrl reducing agents, such as DTT or GSH, to the growth medium (Ohtake and Yabuuchi, 1991, Grant et al., 1996).

Glutathione also constitutes the principal redox buffer in the lumen of the ER (Hwang et al., 1992). In a landmark study, Lodish and colleagues examined the redox state of the tetrapeptide NYTC following equilibration with small molecules in the secretory pathway of mammalian cells. The model peptide contained both an exchangeable cysteine thiol and an N-linked glycosylation site utilized to mark diffusion of the peptide into the ER lumen. In mammalian cells, the disulfide-linked fraction of the glycosylated peptide was found exclusively in mixed-disulfides with glutathione, rather than with cysteine or cysteamine, demonstrating that glutathione serves as the principal redox buffer in the secretory pathway (Hwang et al., 1992). The redox state of glutathione in the lumen of secretory organelles was then calculated based on the equilibrium constant for the formation of NYTC-glutathione mixed-disulfides in vitro. The [GSH]/[GSSG] ratio ranged from 3:1 to 2:1, corresponding to a redox potential of approximately -180 mV (Hwang et al., 1992).

The abundance of GSSG in the ER led to the proposal that GSSG would serve as the immediate source of oxidizing equivalents utilized for protein disulfide bond formation in vivo (Bader et al., 1999b, Hwang et al., 1992). This view was consistent with the finding that GSSG could provide oxidizing equivalents for disulfide bond formation in vitro, by donating disulfide bonds directly to substrate proteins or to folding catalysts such as PDI (Saxena and Wetlaufer, 1970). Further, glutathione redox buffers of similar composition to the redox buffer present in the organelles of the mammalian secretory pathway provided optimal rates for oxidative protein folding in vitro (Lyles and Gilbert, 1991).

Recently, the role of glutathione during oxidative protein folding in vivo has been investigated directly through the use of a yeast mutant devoid of intracellular glutathione. In contrast to the expectation were GSSG important as an oxidant in the yeast ER, the oxidative folding of carboxypeptidase Y (CPY), a vacuolar enzyme with five native disulfide bonds (Endrizzi et al., 1994), proceeds with normal kinetics in *gsh1Δ* mutants (Frand and Kaiser, 1998). This result shows that GSSG does not serve as an obligate intermediate during protein oxidation in vivo. Protein disulfide bond formation in the ER must therefore rely upon a different disulfide donor, such

as Ero1p.

In light of the model that GSSG drives protein disulfide bond formation in the ER, protein oxidation in vivo was thought to depend upon a mechanism dedicated to the concentration of intraluminal GSSG (Hwang et al., 1992). Two mechanisms envisioned were the preferential transport of GSSG into the ER lumen, and the enzymatic production of GSSG inside the ER (Banhegyi et al., 1999). Consistent with the former model, the preferential uptake of GSSG into canine pancreatic microsomes was reported (Hwang et al., 1992). However, the apparent K_m for the presumptive transporter exceeded the reported value for the cytosolic concentration of GSSG by one to two orders of magnitude (Hwang et al., 1992). Utilizing improved methods for the quantification of intraluminal glutathione pools, the preferential transport of GSH, as opposed to GSSG, into rat liver microsomal vesicles has also been observed (Banhegyi et al., 1999). In this case, the apparent K_m for facilitated transport of glutathione was 1.65 mM, consistent with intracellular glutathione concentrations (Banhegyi et al., 1999). In this experimental system, intravesicular GSH was converted to GSSG over time, supporting the view that intraluminal GSSG is generated from GSH by processes within the ER lumen (Banhegyi et al., 1999).

Consistent with this model, the production of oxidized glutathione in yeast cells has recently been linked to the activity of Ero1p (Cuozzo and Kaiser, 1999). Mutation of *ERO1* impedes the recovery of GSSG levels following the treatment of cells with reductant, whereas overproduction of *ERO1* stimulates the recovery of GSSG levels (Cuozzo and Kaiser, 1999). The oxidation of GSH may be coupled to the activity of Ero1p in promoting protein disulfide bond formation if intraluminal GSH becomes oxidized through thiol-disulfide exchange reactions with unstable protein disulfides or oxidized PDI (Cuozzo and Kaiser, 1999). Alternatively, GSH could be oxidized directly by Ero1p. In either case, reduced glutathione transported into the ER would compete with proteins thiols for oxidizing equivalents derived from Ero1p. Consistent with this model, the depletion of intracellular glutathione ameliorates the defects in protein disulfide bond formation caused by the loss of *ERO1* function (Cuozzo and Kaiser, 1999).

Glutathione thus serves as a net reductant in the secretory pathway, as it does in the cytosol (Cuozzo and Kaiser, 1999). The reducing equivalents contributed by GSH during oxidative protein folding in the ER may protect newly-synthesized proteins from the deleterious effects of transiently hyperoxidizing redox conditions, since oxidative protein folding in *gsh1Δ* cells is hypersensitive to the treatment of cells with an exogenous oxidant (Cuozzo and Kaiser, 1999). Glutathione may also facilitate disulfide bond isomerization in the ER, by reducing non-native, unstable protein disulfide bonds, or by reducing PDI, which is needed in dithiol form for catalysis of disulfide isomerization.

PROTEIN DISULFIDE ISOMERASE: A CATALYST FOR THIOL-DISULFIDE EXCHANGE IN THE ER

Protein disulfide isomerase serves as the principal catalyst of disulfide bond formation and isomerization in the ER. This 57 kDa enzyme possesses the domain structure a-b-b'-a'-c, in which the first four domains display structural homology to thioredoxin (Kemmink et al., 1997). The a and a' domains each contain a pair of redox active cysteines in the motif WCGHCK (Edman et al., 1995). In vitro, the active-site cysteines of PDI can participate in thiol-disulfide exchange reactions catalyzing dithiol oxidation, disulfide reduction, or disulfide isomerization, depending on the nature of the substrate protein and the redox conditions of the assay (reviewed by Freedman et al., 1984). PDI also engages in thiol-disulfide exchange reactions with newly-synthesized secretory proteins in the yeast ER, as demonstrated by the capture of mixed-disulfides between PDI and the ER precursor of CPY (Frand and Kaiser, in press).

The *PDI1* gene of yeast is essential for cell viability, and for oxidative folding of CPY (Tachibana and Stevens, 1992, Lamantia and Lennarz, 1993). A CGHS-CGHS active-site mutant of PDI, which retains isomerase activity but lacks detectable oxidase or reductase activity in vitro, can rescue the inviability of cells with a chromosomal deletion of *PDI1*, indicating that the isomerase activity of PDI is

essential (Laboissière et al., 1995).

Nevertheless, recent findings have shown that PDI also plays an extensive and critical role as an oxidase in vivo. PDI is found predominantly in the oxidized form in wild-type cells, rather than in the reduced (dithiol) form suited for catalysis of disulfide shuffling (Frand and Kaiser, in press). Moreover, the defect in CPY maturation associated with depletion of PDI can be traced to a defect in protein oxidation (Frand and Kaiser, in press). Mutations in the internal residues of the active-sites of PDI also render cells hypersensitive to the reductant DTT. This phenotype may stem from a deficiency in oxidase activity in vivo, since these mutations are likely to lower the redox potential of PDI (Holst et al., 1997, Chivers et al., 1997). Further, although *pdi1Δ* cells rescued by overproduction of CGHS-CGHS PDI are viable, this strain grows slowly and remains hypersensitive to DTT. Complete rescue of *pdi1Δ* cells requires a redox-active, CXXC motif (Holst et al., 1997). Although the oxidase activity of PDI is thus clearly important for optimal growth and secretion, it may not be essential for cell viability if alternative, albeit less efficient, sources of oxidase activity in the ER can support the formation of disulfide bonds in essential secretory proteins.

HOMOLOGS OF PDI IN THE YEAST ER

In addition to *PDI1*, there are four genes in Saccharomyces cerevisiae predicted to encode thioredoxin-like oxidoreductases localized to the ER. These genes include *MPD1* (for multicopy suppressor of *PDI1* deletion), *MPD2*, *EUG1* (ER protein unnecessary for growth), and the as yet uncharacterized ORF YIL005w (Tachikawa et al., 1995, Tachikawa et al., 1997, Tachibana and Stevens, 1992 Cherry et al., 1997). Mpd1p, Mpd2p, and the predicted product of YIL005w each contain one redox-active CXXC motif, while Eug1p contains two active-sites with the sequences CLHS and CIHS (Tachikawa et al., 1995, Tachikawa et al., 1997, Cherry et. al, 1997, Tachibana and Stevens, 1992). Consistent with an ER localization, these enzymes each possess an N-terminal signal sequence, multiple acceptor sites for N-

linked glycosylation, and a C-terminal HDEL motif mediating ER retention (Pelham et al., 1988). Only the predicted product of YIL005w lacks an HDEL motif, however this protein may contain two transmembrane domains that could also mediate ER retention. The expression of *MPD1*, *MPD2*, and *EUG1* is induced by the Unfolded Protein Response upon the accumulation of misfolded proteins in the ER (Cox et al., 1993, Khono et. al., 1993).

Broad functional redundancy among the PDI-homologs was revealed by the isolation of *MPD1*, *MPD2*, and *EUG1* as high copy suppressors of the inviability associated with chromosomal deletion of *PDI1*. Overexpression of *MPD1*, *MPD2*, or *EUG1* also partially restores the oxidative folding of CPY in *pdi1Δ* cells (Tachikawa et al., 1995, Tachikawa et al., 1997, Tachibana and Stevens, 1992).

Together, this family of oxidoreductases may be responsible for catalyzing both the isomerization and the oxidation of newly synthesized secretory proteins. In wild-type cells, these enzymes may function in a highly redundant manner. Alternatively, each of these enzymes may be dedicated to the catalysis of disulfide shuffling, dithiol oxidation, or disulfide reduction. For example, the absence of a C-terminal cysteine in both active-sites of Eug1p would be predicted to restrict Eug1p to the catalysis of disulfide reshuffling. The PDI homologs could also interact with specific sets of substrate proteins. Genetic dissection of the function of these PDI homologs has proven complex, in part because deletion of *MPD1*, *MPD2*, or *EUG1* does not interfere with cell growth or oxidative protein folding (Tachikawa et al., 1995, Tachikawa et al., 1997, Tachibana and Stevens, 1992). However, phenotypes associated with loss-of-function mutations in any of these genes may be obscured by compensatory induction of the remaining oxidoreductases with the UPR. Phenotypes associated with overexpression of these genes must also be interpreted with caution, since thioredoxin-like oxidoreductases can display significant functional plasticity when overproduced in altered redox environments (Rietsch et al., 1997, Stewart et al., 1998). The biochemical properties of these enzymes, including their redox potential and their oxidase or isomerase activity in vitro remain to be assessed.

MAMMALIAN HOMOLOGS OF PDI

The proliferation of PDI homologs observed in the yeast ER is also observed in mammalian cells, where PDI homologs including ERp57/GRP58, ERp72, PDIp, and P5 have been implicated in multiple aspects of ER protein maturation, including not only the oxidative folding of specific secretory proteins, but also the assembly of multiprotein complexes and protein degradation (reviewed by Ferrari and Söling, 1999). As an example, ERp57, which possesses isomerase activity in vitro, has been shown to associate indirectly with monoglycosylated ER proteins, via clanexin or calreticulin (Zapun et al., 1998). Through such a tripartite interaction, ERp57 may facilitate the assembly of newly-synthesized MHC class I molecules (Lindquist et al., 1998).

Mammalian PDI has further been implicated in several non-redox processes associated with protein maturation in the ER. PDI serves as the β-subunit of the heterotetrameric enzyme prolyl hydroxylase, which is responsible for catalyzing prolyl hydroxylation of the pro-alpha chains of procollagen. PDI is also the β-subunit of the heterodimeric microsomal triglyceride transfer complex (Pihlajaniemi et al., 1987, Wetterau et al., 1990).

ACTIVITIES OF PDI IN VITRO AND IN VIVO

Anfinsen and colleagues first isolated PDI from rat liver microsomes as an activity catalyzing the reactivation of reduced, denatured ribonuclease A (RNase A) (Goldberger et al., 1963). Since sulfhydrl oxidation was not limiting in this assay, the authors suggested that PDI served as a catalyst for disulfide interchange, rather than for protein oxidation, in vivo (Givol et al., 1964). Since then, assays for PDI catalysis of oxidative refolding have typically employed redox buffers containing both GSH and GSSG. The rate of oxidative refolding always depends upon the redox state of the buffer, expressed as the $[GSH]^2/[GSSG]$ ratio (Lyles and Gilbert, 1991).

The premiere assays for PDI-catalysis of protein oxidation monitor the reactivation of reduced RNase A or BPTI (bovine pancreatic trypsin inhibitor), while assays for PDI-catalysis of disulfide isomerization monitor the reactivation of misoxidized, denatured RNase A or partially-reduced BPTI (Goldberger et al., 1963, Givol et al., 1965, Weissman and Kim, 1993).

In vitro, PDI affords relatively modest overall rate enhancements for oxidative refolding, partly due to the low turnover number (0.5-1 min^{-1}) of the enzyme and its relatively high K_m values for substrate proteins (5-20 μM) (Gilbert, 1998). However, the high concentration of PDI relative to most folding substrates in the ER may enhance the overall utility of the enzyme in vivo (Zapun et al., 1992, Gilbert, 1998).

In vitro, those redox buffers affording maximal rates for oxidative protein folding also favor the complete reduction of PDI, and the reduced (dithiol) form of PDI therefore appears largely responsible for the catalytic activity of the enzyme. Since the dithiol form of PDI is suited for catalysis of disulfide reshuffling, this observation has been interpreted as additional evidence that isomerization is the main function of PDI in vivo. Consistent with this view, PDI serves as a highly efficient catalyst for the rate-limiting disulfide rearrangements characteristic of BPTI refolding. PDI provides a 3,000 to 6,000-fold enhancement in the rate of conversion of kinetically trapped, partially oxidized BPTI intermediates to the native enzyme (Weissman and Kim, 1993).

The ability of PDI to serve as a direct oxidant for substrate proteins in vitro is limited by the rate of re-oxidation of PDI by GSSG (Westphal et al., 1998). Indeed, the use of buffers with too high of a GSSG concentration often leads to the production of unproductive mixed-disulfides between glutathione and the substrate protein or PDI, and thereby inhibits native disulfide bond formation in the substrate (Gilbert, 1998). Nevertheless, oxidized PDI, purified from mammalian cells, can stochiometrically transfer oxidizing equivalents to RNase A, in the absence of a redox buffer (Lyles and Gilbert, 1991b).

The presence of GSSG in the ER led to the supposition that in vivo the oxidase activity of PDI would be coupled to re-oxidation of the enzyme by GSSG, as

it is in vitro. The redox state of PDI in vivo would then be determined by equilibration with glutathione. Since the redox potential of the catalytic disulfide of PDI (-110 to -180 mV) is roughly equivalent to the redox potential of the mammalian ER (Lundström and Holmgren, 1993, Hwang et al., 1992), PDI would then exist as an equimolar mixture of the reduced and oxidized forms. However, direct assessment of the redox state of PDI in vivo has revealed that the vast majority of PDI is maintained in the oxidized form in a manner dependent upon Ero1p (Frand and Kaiser, in press). Although the formal possibility exists that the redox state of glutathione in the yeast ER differs significantly from that of glutathione in the mammalian secretory pathway, this observation suggests that PDI is in fact not in equilibrium with glutathione in vivo. Rather, PDI may be maintained in the oxidized form by rapid and specific thiol-disulfide exchange with Ero1p (Frand and Kaiser, in press).

The mechanism of PDI-catalysis of disulfide bond isomerization involves the nucleophilic attack upon a non-native or unstable disulfide bond in a substrate protein by a thiolate anion derived from the N-terminal cysteine of an active-site of PDI. This reaction generates a mixed-disulfide intermediate between PDI and the substrate protein, and simultaneously liberates a potentially reactive thiolate anion in the substrate. The latter thiolate is then thought to initiate a series of intramolecular thiol-disulfide exchange reactions culminating in the attack of the peptide-PDI mixed-disulfide bond by a second thiolate anion derived from the substrate (Walker et al., 1996, Walker and Gilbert, 1997). Through this series of intramolecular thiol-disulfide exchange reactions, the substrate protein would acquire a new complement of disulfide bonds, and PDI would ultimately be released still in the reduced form. Theoretically, this "scanning" mechanism would be driven by the search for the most thermodynamically stable substrate conformation (Walker and Gilbert, 1997). The observation that the redox-inactive CGHS-CGHS active-site mutant of PDI retains full isomerase activity towards BPTI is consistent with the scanning model for isomerization, since this mechanism obviates the need for oxidation of PDI (Laboissière et al., 1995). However, CGHS-CGHS PDI displays only 5% of the

isomerase activity associated with wild-type PDI towards scrambled RNase A. In the context of the scanning model, this observation was interpreted as evidence that the C-terminal active-site cysteines of PDI may normally help release PDI from unproductive mixed-disulfides with substrates lacking thiols of sufficient reactivity to attack the PDI-substrate mixed disulfide bond (Walker et al., 1996, Walker and Gilbert, 1997).

Disulfide bond isomerization could, theoretically, also proceed through the reductive breakage and subsequent re-oxidation of protein disulfide bonds. The observation that an influx of reducing equivalents is required to sustain the catalytic activity of the E. coli disulfide isomerase, DsbC, suggests that isomerization may proceed via the latter mechanism in vivo (Rietsch et al., 1997). In this case, CGHS-CGHS PDI would be predicted to display diminished isomerase activity. The decreased isomerase activity of CGHS-CGHS PDI towards scrambled RNase A may thus suggest that disulfide rearrangements in this substrate proceed via cycles of reduction and re-oxidation.

Both in vitro and in vivo, PDI binds peptides with broad specificity, and in a manner independent of the oxidoreductase activity of the enzyme (Noiva et al., 1993). In mammalian cells, newly-translocated secretory proteins, immunoglobulins, and misfolded proteins retained in the ER have all been chemically crosslinked to PDI. In vitro, radiolabeled peptides have been crosslinked to human PDI, and peptides serve as competitive inhibitors PDI's oxidoreductase activity (Klappa et al., 1997, Otsu et al., 1994, Roth and Pierce, 1987, Noiva et al., 1991, Klappa et al., 1995, Morjana and Gilbert, 1991). The most important parameter identified thus far in determining the affinity of a peptide for PDI appears to be its length, since PDI binds heptapeptides with 1,000-fold higher affinity than tetrapeptides (Westphal et al., 1998, Morjana and Gilbert, 1991). In vitro, cysteine residues can increase the affinity of a model peptide for PDI 4-8 fold, and hydrophobic residues in the proximity of a reactive cysteine may enhance the binding of a peptide to PDI nearly 10-fold (Morjana and Gilbert, 1991, Noiva et al., 1993, Westphal et al., 1998, Klappa et al., 1997). The length restriction for high-affinity binding to PDI may in part explain why

the tripeptide glutathione appears to serve as a poor substrate for the enzyme (Westphal et al., 1998). In human PDI, the b' domain appears essential but not always sufficient for substrate binding (Klappa et al., 1998, Noiva et al., 1993).

Peptide binding may contribute to the catalytic effectiveness of PDI in vitro, since neither of the two thioredoxin-like domains of PDI alone displays substantial isomerase activity relative to the full-length enzyme (Darby and Creighton, 1995). However, peptide binding must be dispensable for the essential function of PDI in yeast, since active-site mutants of E. coli thioredoxin (Trx) can rescue the inviability of *pdi1Δ* cells, even though Trx lacks a peptide binding domain and fails to bind peptides in vitro (Chivers et al., 1996, Noiva et al., 1993).

PDI further displays both chaperone and anti-chaperone activities during the oxidative refolding of certain substrates, such as lysozyme and immunoglobulins (Puig and Gilbert, 1994, Lilie et al., 1994). In the absence of PDI, approximately 50% of reduced, denatured lysozyme aggregates, while 50% re-folds to yield the active enzyme. Catalytic quantities of PDI enhance the rate of folding of the latter population of molecules. However, when high concentrations of lysozyme are present in vitro, greater than catalytic quantities of PDI stimulate aggregation of the substrate, and thereby decrease the yield of active lysozyme. In contrast, greater than stochiometric quantities of PDI prevent aggregation of the substrate, and increase the yield of active lysozyme (Gilbert, 1998). These phenomena may both be attributable to a bivalent binding interaction between PDI and lysozyme, since sub-stochiometric quantities of PDI would then promote extensive crosslinking of lysozyme aggregates, whereas super-stochiometric quantities of PDI would serve to solubilize additional lysozyme molecules (Gilbert, 1998). These anti-chaperone and chaperone activities appear dependent on peptide binding, but not on the oxidoreductase activity of PDI (Puig et al., 1994). The physiologic relevance of the anti-chaperone activity of PDI remains unclear, in part because PDI may normally be present in greater than stochiometric quantities relative to most secretory proteins in the ER (Zapun et al., 1992).

DISULFIDE BOND FORMATION IN THE E. COLI PERIPLASM

In the E. coli periplasm, oxidative protein folding proceeds in the absence of small molecule intermediates such as glutathione. Rather, protein disulfide bond formation is catalyzed through a series of thiol-disulfide exchange reactions mediated entirely by periplasmic oxidoreductases.

DsbA serves as the principal oxidant of newly-translocated proteins in the periplasm (Bardwell et al., 1991). This soluble, 22 kDa thiol-disulfide oxidoreductase contains the active-site CPHC in a domain with structural homology to thioredoxin (Martin et al., 1993). Mutation of dsbA disrupts the efficient formation of disulfide bonds in numerous substrate proteins, including alkaline phosphatase, OmpA, and the flagellar P-ring protein, FigI (Bardwell et al., 1991, Kamitami et al., 1992, Daily and Berg, 1993). As a consequence of the loss of activities normally associated with these and other proteins, dsbA mutants display pleiotropic phenotypes, including immotility (Daily and Berg, 1993). Mutants of dsbA are also hypersensitive to the reductant DTT (Missiakas et al., 1993).

Interestingly, dsbA was first identified in a screen for mutants defective in the insertion into the cytoplasmic membrane of a MalF-β-galactoside fusion protein. The dsbA mutant may have answered this screen as a consequence of preventing disulfide bond formation in β-galactosidase segments exposed to the periplasm. Disrupting disulfide bond formation in these segments may have facilitated re-entry of the fusion protein into the cytosol, allowing recovery of β-galactosidase activity (Bardwell et al., 1991).

In vitro, stochiometric quantities of DsbA prove remarkably efficient in transferring oxidizing equivalents to substrate proteins (Zapun and Creighton, 1994). The oxidizing power of DsbA stems from the high redox potential of its catalytic disulfide (-89 to -120 mV), and the unusually low pK_a (3.5) of its N-terminal, active-site cysteine side-chain (Zapun et al., 1993, Wunderlich and Glockshuber, 1993,Grauschopf et al., 1995). Both properties contribute to the relative instability of the disulfide, versus the dithiol, form of DsbA. The equilibrium constant for oxidation

of DsbA by glutathione is a mere 80 μM, a value up to ten orders of magnitude below that observed for structural protein disulfide bonds (Zapun et al., 1993). Further, oxidized DsbA undergoes thiol-disulfide exchange reactions with reduced glutathione at a rate 1,000 fold faster than a typical protein disulfide. Substrate binding may also enhance the catalytic activity of DsbA, since the enzyme reacts up to 1,000 fold faster with a model peptide than with glutathione (Darby and Creighton, 1995b).

The sustained activity of DsbA as an oxidase requires regeneration of the disulfide form of the enzyme. Several lines of evidence indicate that the cytosolic membrane protein DsB is responsible for re-oxidation of DsbA in vivo (Bardwell et al., 1993, Daily and Berg, 1993, Missiakas et al., 1993). While DsbA is oxidized in wild-type cells, DsbA is found in the reduced form in a dsbB mutant (Kobayashi et al., 1997, Bardwell et al., 1993). Thiol-disulfide exchange between DsbA and DsbB has been indicated by the capture of mixed-disulfides between the two proteins following the treatment of bacterial cells with acid (Kishigami et al., 1995 Guilhot et al., 1995). Moreover, the oxidation of DsbA by DsbB has been reconstituted in vitro, utilizing purified DsbA and membrane preparations containing catalytic quantities of DsbB (Bader et al., 1997). In this system, The K_m for binding of DsbA to DsbB is 10 μM, a value consistent with the physiologic concentration of DsbA (Bader et al., 1997).

Several properties of DsbB indicate that the enzyme mediates the net flux of oxidizing equivalents into the DsbA-dependent pathway for protein oxidation in the periplasm. Mutants in dsbB display defects in the oxidative folding of periplasmic marker proteins and are hypersensitive to DTT (Bardwell et al., 1993, Missiakas et al., 1993, Daily and Berg, 1993). However, these phenotypes can be suppressed by the addition of an exogenous thiol oxidant, such as cystine or GSSG, to the growth medium (Bardwell et al., 1993, Daily and Berg, 1993). Moreover, overexpression of DsB confers resistance to otherwise toxic levels of DTT (Missiakas et al., 1993).

The activity of DsbB, both in vitro and in vivo, requires two redox-active disulfide bonds (Jander et al., 1994, Bader et al., 1999). The cysteine pairs that

form these bonds define positions 41-44 and 104-130 of the primary DsbB sequence, and occupy two separate periplasmic loops of the enzyme (Jander et al., 1994). Both cysteine pairs are found in the oxidized form in vivo (Kobayashi and Ito, 1999).

The Cys104-Cys130 disulfide bond of DsbB is thought to engage directly in thiol-disulfide exchange with DsbA, since a substitution mutant of DsbB in which Cys104 is replaced with serine is completely defective in the formation of mixed-disulfides with DsbA33S (Guilhot et al., 1995, Kishigami et al., 1996).
The Cys41-Cys44 disulfide is thought to re-oxidize the Cys104-Cys130 cysteine pair, since mutational inactivation of the Cys41-Cys44 disulfide results in reduction of Cys104 and Cys130 in vivo (Kobayashi and Ito, 1999, Guilhot et al., 1995, Kishigami et al., 1996). In contrast, the oxidized redox state of the Cys41-Cys44 cysteine pair of DsbB is impervious to mutational inactivation of the Cys104-Cys130 disulfide, and is further recalcitrant to the treatment of cells with up to 10 mM DTT (Kobayashi and Ito, 1999). Verification of this model for DsbB function awaits documentation of intramolecular thiol-disulfide exchange between Cys41-Cys44 and Cys104-Cys130 of DsbB.

Re-oxidation of DsbB is accomplished by the transfer of electrons to molecular oxygen or alternate acceptors via the respiratory chain (Kobayashi et al., 1997, Bader et al., 1998, Kobayashi and Ito, 1999, Bader et al., 1999). In the in vitro system for DsbB-catalyzed oxidation of DsbA, electrons flow from DsbB to molecular oxygen via ubiquinone and either cytochrome bd or cytochrome bo oxidase (Bader et al., 1998, Bader et al., 1999). These heme-containing proteins represent the two terminal oxidases in E. coli, conducting electron flow from intracellular pools of ubiquinone to molecular oxygen under aerobic growth conditions. Menaquinone serves as the intermediate in electron transfer to alternate acceptors under anaerobic growth conditions, and menadione can serve as an immediate electron acceptor for DsbB in vitro, albeit less efficiently than derivatives of ubiquinone (Bader et al., 1999). Consistent with these findings, the depletion of intracellular pools of heme, or ubiquinone and menaquinone promotes the facile reduction of the

Cys41-Cys44 disulfide bond of DsbB (Kobayashi and Ito, 1999). Depletion of these metabolites is further correlated with the reduction of DsbA and the eventual accumulation of DsbB-DsbA mixed disulfides (Kobayashi et al., 1997, Kobayashi and Ito, 1999). Moreover, protoheme deprivation disrupts disulfide bond formation in periplasmic β-lactamase (Kobayashi et al., 1997). Together, these results position DsbB as the link between protein disulfide bond formation and electron transport systems mediating general cellular metabolism.

Mammalian PDI partially complements the E. coli dsbA mutant, suggesting functional conservation of the periplasmic pathway for disulfide bond formation in the mammalian ER (Humphreys et al., 1995). Yeast Ero1p shares several properties with E. coli DsbB, further implying an extensive conservation of the DsbB-DsbA system.

Alongside the DsbB-DsbA dependent pathway for protein oxidation in the bacterial periplasm, an analogous pathway operates to insure the efficient isomerization of non-native protein disulfide bonds. Two additional thiol-disulfide oxidoreductases, the soluble enzyme DsbC and the cytoplasmic membrane protein DsbD, function in this pathway. DsbC appears to serve as the principal periplasmic isomerase, since mutation of dsbC impairs the oxidative folding of periplasmic proteins with multiple or inappropriate disulfide bonds, including heterologously expressed mouse urokinase, alkaline phosphatase, and an alkaline phosphatase mutant with non-native disulfide bonds (Rietsch et al., 1996, Sone et al., 1997, Rietsch et al., 1997). In contrast, mutation of dsbC does not interfere with the oxidative folding of substrates containing only a single disulfide bond, such as OmpA (Rietsch et al., 1996). DsbC also serves as an efficient isomerase in vitro, catalyzing the oxidative refolding of misoxidized BPTI intermediates (Zapun et al., 1995). The activity of DsbC in vitro is enhanced significantly upon the binding of a substrate protein (Darby et al., 1998). An additional periplasmic oxidoreductase, DsbG may operate as an isomerase in parallel to DsbC (Bessette et al., 1999).

As predicted for an enzyme dedicated to the catalysis of disulfide reshuffling, the active-site cysteines of DsbC are found in the reduced form in vivo (Joly and

Swartz, 1997, Rietsch et al., 1997). Maintenance of the reduced redox state of DsbC requires DsbD, an oxidoreductase localized to the cytoplasmic membrane (Rietsch et al., 1996, Missiakas et al., 1995). DsbD serves as an intermediate in the transfer of electrons from cytoplasmic thioredoxin to DsbC (Rietsch et al. 1997). Consistent with this model, mutations in the genes encoding thioredoxin, thioredoxin reductase, and DsbD are each associated with defects in the maturation of periplasmic proteins with multiple disulfide bonds (Rietsch et al., 1997). The observation that oxidative folding of proteins with multiple disulfide bonds requires a source of reducing equivalents in the E. coli periplasm suggests that catalysis by DsbC may involve the reductive breakage of inappropriate disulfide bonds with concomitant oxidation of DsbC.

CATALYSIS OF DISULFIDE BOND FORMATION BY MEMBERS OF THE THIOREDOXIN SUPERFAMILY

Oxidative protein folding is catalyzed both in vitro and in vivo by thiol-disulfide oxidoreductases of the thioredoxin superfamily. The prototype for this family, thioredoxin, is a 12 kDa cytosolic enzyme with the active-site CGPC responsible for reducing the catalytic disulfide of ribonucleotide reductase as well as other protein disulfides (Holmgren, 1968, Martin et al., 1995). Members of the thioredoxin superfamily include DsbA and DsbC from E. coli as well as PDI from eukaryotes. These enzymes each possess at least one domain that displays sequence or structural homology to thioredoxin and contains a pair of redox-active cysteines in the motif CXXC (Martin et al., 1993, Edman et al., 1985, Shevchick, et al., 1994).

The universal catalytic mechanism of these enzymes involves the formation of a mixed-disulfide bond between the N-terminal active-site cysteine and the substrate protein (Gilbert, 1994). Substrate reduction begins with the nucleophilic attack of a substrate disulfide by the thiolate anion derived from the N-terminal active-site cysteine of the enzyme. The resulting mixed-disulfide can be resolved by intramolecular attack of the mixed-disulfide bond by a thiolate anion derived from the

C-terminal active-site cysteine. In the presence of a glutathione redox buffer, the enzyme-substrate mixed-disulfide can also be resolved via intermolecular thiol-disulfide exchange with GSH. The extent to which this reaction competes with intramolecular thiol-disulfide exchange will depend in part upon the effective molarity of the enzyme's active-site cysteines and in part upon the GSH concentration (Gilbert, 1998). Following reduction of the substrate, the oxidoreductase is released in disulfide form, necessitating reduction of the catalyst for continued activity. Consistent with this mechanism, the N-terminal active-site cysteine of a thioredoxin-like oxidoreductases is essential for catalysis of thiol-disulfide exchange (Walker et al., 1996, Laboissière et al., 1995).

Two interrelated properties of the CXXC motif influence the catalytic activity of a thioredoxin-like oxidoreductase; the standard redox potential (E'_o) of its catalytic disulfide, and the pK_a of its N-terminal active-site cysteine side chain (Chivers et al., 1997). The standard redox potential provides an indication of the relative stability of the dithiol versus the disulfide form of the enzyme. Increasing the redox potential of an oxidoreductase will thus increase the tendency of the enzyme to transfer its disulfide bond to a substrate protein. In vivo, enzymes with a relatively high redox potential tend to function as oxidants, whereas enzymes with a relatively low redox potential, such as thioredoxin (E'_o = -270 mV), tend to function as reductants (Åslund et al., 1997). DsbA is the most oxidizing member of the thioredoxin superfamily yet characterized, with a standard redox potential of -89 to -120 mV (Wunderlich and Glockshuber, 1993, Zapun et al., 1993). The redox potential of PDI falls between these two extremes, at -110 to -180 mV (Lundström and Holmgren 1993).

Reaction rates for thiol-disulfide exchange are further influenced by the pK_a of the enzymic cysteine, a value reflecting the relative stability of the thiol versus thiolate anion forms of the cysteine side chain. Cysteines with low pK_a values generally serve as good leaving groups, but poor nucleophiles during thiol-disulfide exchange reactions. The N-terminal cysteines found in the CXXC motifs of thioredoxin-like oxidoreductases display pK_a values lower than that observed for typical protein thiols (pK_a of 8.7). For example, the pK_a of the reactive cysteines in

mammalian PDI is 6.7 (Hawkins and Freedman, 1991). The reactive cysteine of DsbA possesses an extremely low pK_a of 3.5 (Nelson and Creighton, 1994). This low pK_a ensures that the reactive cysteine of DsbA will be the preferred leaving group from DsbA-substrate mixed-disulfides. Moreover, the depressed pK_a of this cysteine side chain destabilizes the catalytic disulfide bond of DsbA, and thus contributes significantly to the high redox potential and remarkable oxidizing power of the enzyme (Grauschopf, et al., 1995). The pK_a values for the reactive cysteines of DsbA and PDI seem well-suited for the catalysis of thiol-disulfide exchange in their respective physiologic environments, as the bacterial periplasm is more acidic than the lumen of the ER.

The redox potential of a thioredoxin-like oxidoreductase is determined in large part by the identity of the intervening amino-acids of the CXXC motif. Replacement of the CXXC motif of a relatively reducing enzyme with the CXXC motif from an oxidizing one will confer a higher redox potential upon the recipient enzyme, and vice-versa (Chivers et al., 1996, Mössner et al., 1998, Jonda et al., 1999, Huber-Wunderlich and Glockshuber, 1998). Alteration of the intervening residues of the CXXC motif of DsbA or PDI can thus diminish the redox potential of the enzyme (Grauschopf et al., 1995, Holst et al., 1997).

The physiologic significance of the redox potential of DsbA has been investigated by testing variants of thioredoxin and DsbA for complementation of the dsbA mutant. Thioredoxin (Trx) localized to the periplasm can restore efficient disulfide bond formation in the dsbA mutant, but only after the active site of the enzyme is altered to have a higher redox potential (-221 to -195 mV) (Jonda et al., 1999). Wild-type Trx confers only weak complementing activity (Debarbieux and Beckwith, 1998). The complementing activity of a thioredoxin variant correlates with the oxidase activity of the enzyme in vitro, indicating that a catalyst of sufficiently high redox potential to support the rapid and complete oxidation of substrate proteins is required for efficient disulfide bond formation in vivo (Jonda et al., 1999). All of the Trx variants, including the wild-type enzyme, were efficiently re-oxidized by DsbB (Jonda et al., 1999, Debarbieux and Beckwith, 1998).

Thioredoxin localized to the yeast ER can also rescue the inviability of cells with a chromosomal deletion of *PDI1*, but only after the active site of the enzyme is altered to have a higher redox potential (-235 to -200 mV) and a lower p*Ka* (Chivers et al., 1996, Chivers et al., 1997). These changes should increase the intraluminal concentration of the thiolate anion derived from the Trx reactive cysteine, and may therefore enhance the isomerase activity of the enzyme in vivo. The rescuing activity of modified Trx was attributed to this increase in thiolate anion concentration in light of the view that PDI serves predominantly as an isomerase in living cells (Chivers et al., 1996). However, increasing the redox potential of Trx will also enable the enzyme to serve as a more efficient oxidase in vivo, and this change could contribute to the rescuing activity of modified Trx.

The catalytic activity of all thioredoxin-like oxidoreductases depends upon the redox state of their environment. Regardless of their physiologic function, these enzymes will catalyze dithiol oxidation, disulfide reduction, or disulfide isomerization in vitro given an appropriate redox buffer. For example, reduced DsbA can serve as an isomerase in vitro although the enzyme is a potent and specific catalyst for dithiol oxidation in the E. coli periplasm (Joly and Swartz, 1994, Bardwell et al., 1991). The expression of these enzymes in ectopic or perturbed redox environments in vivo may also influence their activity, within certain limits. For example, thioredoxin will catalyze disulfide bond formation if the redox state of the cytosol becomes abnormally oxidizing, even though Trx normally serves as a reductase (Stewart et al., 1998). Likewise, DsbC may serve as an oxidase for newly-translocated periplasmic proteins in the absence of DsbD, even though DsbC normally functions as an isomerase (Rietsch et al., 1997). DsbC may also serve as an oxidase simply when overexpressed (Shevchick et al., 1994). The functional plasticity observed among these oxidoreductases highlights their broad substrate compatibility and enzymatic versatility.

PATHWAYS FOR OXIDATIVE PROTEIN FOLDING IN VITRO

Mechanisms governing the formation of native protein disulfide bonds in vitro have been studied almost exclusively by monitoring the oxidative refolding of two small proteins, bovine pancreatic trypsin inhibitor (BPTI), and ribonuclease A (RNase A). To achieve reasonably efficient rates for oxidative protein folding in vitro, a redox buffer must be employed containing both oxidizing and reducing equivalents, typically provided by GSSG and GSH (Saxena and Wetlaufer, 1970). The oxidizing equivalents in GSSG support protein disulfide bond formation, while the reducing equivalents in GSH allow for reduction of those disulfides prohibitive to the acquisition of native structure. The transfer of redox equivalents from the buffer to the substrate may occur directly, or via a catalyst, such as PDI (Saxena and Wetlaufer, 1970). The rate of oxidative refolding in vitro, regardless of the presence of a catalyst, depends upon the redox state of the buffer, expressed as the ratio $[GSH]^2/[GSSG]$ (Lyles and Gilbert, 1991). For example, the optimum rate for refolding of reduced RNase A is observed with 1 mM [GSH] and 0.2 mM [GSSG] (Lyles and Gilbert, 1991). At equilibrium, the distribution of folding intermediates with the same number of disulfide bonds will depend upon the thermodynamic stability of those species, while the overall distribution of intermediates with different numbers of disulfide bonds will depend upon the redox state of the buffer (Gilbert, 1994).

A pathway for the oxidative folding of BPTI has been reconstructed by monitoring the accumulation of intermediates during refolding of the reduced, denatured protein. Quasi-native folding intermediates predominate this pathway, to the extent that kinetic traps are created which impede substrate oxidation. BPTI contains three disulfide bonds, two of which are buried in the core of the native protein ([30-51] and [5-55]), and one of which ([14-38]) is exposed to solvent. During the oxidative refolding of reduced, denatured BPTI at pH 8.7, redox isomers with a single disulfide bond rapidly equilibrate to yield two predominant species, each containing one of the core, native disulfides, either [30-51] or [5-55] (Weissman and

Kim, 1991, Creighton, 1977). The predominance of [30-51] and [5-55] is consistent with the existence of native structural elements in these species (Weissman and Kim, 1991, Creighton, 1977). Refolding at pH 7.3 increases the abundance of [5-55], since protonation of the thiols on Cys30 and Cys51 facilitates shielding of these residues in the quasi-native core (Weissman and Kim, 1991). A third intermediate containing the native disulfide [14-38] is also detected during refolding at neutral pH (Dandlez and Kim, 1995).

In the kinetically preferred pathway for BPTI refolding, the two predominant single-disulfide redox isomers are rapidly oxidized to generate the intermediates [30-5114-38] and [5-55 14-38], designated N' and N*, respectively (Weissman and Kim, 1991, Creighton, 1992). Native structure in these intermediates buries those cysteines that would normally form the second core disulfide, prohibiting their oxidation (Weissman and Kim, 1995, Weissman and Kim, 1991). Acquisition of the fully oxidized, native conformation of BPTI requires rearrangement of these intermediates to the species [30-51 5-55], which can be rapidly oxidized to yield native BPTI. The less-stable species [30-51 5-38] and [30-51 5-14], which contain non-native disulfides, serve as obligate intermediates in rearrangement of N and N* (Weissman and Kim, 1991, Creighton, 1992). Consequently, these rearrangements are rate limiting for the overall folding of BPTI. PDI greatly facilitates the resolution of these kinetic traps during the refolding of BPTI, by catalyzing isomerization of N' and N* to the [30-51 5-55] intermediate (Weissman and Kim, 1993). The rate of conversion of N' and N* to native BPTI is increased 3,000-6,000 fold in the presence of PDI, thereby rescuing N* from an otherwise dead-end fate.

β-lactamase encounters a similar kinetic trap during oxidative refolding. This substrate possesses only one native disulfide bond that is buried in the protein's core, but is dispensable for folding. In vitro, the folding of β-lactamase competes with oxidation, resulting in the accumulation of folded but reduced molecules. Conversion of these molecules to native β-lactamase requires a rate-limiting unfolding step to allow for oxidation of the buried cysteines (Frech et al., 1996).

Unlike the situation observed with BPTI, predominant intermediates with

significant native structure have not been observed to accumulate during the oxidative refolding of RNase A, which possesses four native disulfide bonds (Gilbert, 1994). The distribution of single-disulfide RNase A intermediates matches that predicted from a statistical calculation based only on the distances between cysteine residues (Konishi, et al., 1981, Creighton et al., 1977). The appearance of intermediates with three and four disulfide bonds precedes the recovery of enzymatic activity, suggesting a requirement for disulfide rearrangements in the final, possibly rate-limiting, steps of RNase A refolding (Creighton, 1979). However, a single kinetically preferred pathway for the refolding of reduced RNase A has yet to be defined (Konishi, et al., 1982).

How similar are the pathways for oxidative protein folding in vitro and in vivo? For the β-subunit of human chorionic gonadotropin, the pathway for refolding in vitro recapitulates the pathway for maturation of the protein in vivo (Huth et al., 1992, Huth et al., 1992b). Intermediates with non-native disulfide bonds have not been observed during oxidative folding of the influenza HA protein or human chronic gonadotropin in mammalian cells. Such species could nevertheless represent important kinetic intermediates during oxidative folding in vivo (Segal et al., 1992, Huth et al., 1992b). However, increases in the rate of protein oxidation afforded by PDI or DsbA-catalysis may diminish the significance of kinetic traps during oxidative folding in vivo (Gilbert, 1998, Frech et al., 1996). One potential difference between the pathways for oxidative protein folding in vitro and in vivo arises from the potential for disulfide bond formation to initiate co-translationally in living cells. The folding of N-terminal domains could, theoretically, impede the formation of intramolecular disulfides linking distal cysteines (Gilbert, 1994). In yeast cells, the secretion efficiency of heterologous BPTI mutants unable to form native disulfide bonds correlates with the thermodynamic stability of the mutant protein (Kowalski et al., 1998). The added thermodynamic stability afforded by native disulfide bonds may thus facilitate the escape of secretory proteins from quality control mechanisms operating the ER (Kowalski et al., 1998).

TOOLS FOR THE ANALYSIS OF DISULFIDE BOND FORMATION IN LIVING CELLS

The experiments described in this thesis rely extensively upon cell biological methods for manipulation of the intracellular redox state and inhibition of thiol-disulfide exchange in vivo. A description of these methods has been included in this introduction to facilitate the critical evaluation of experiments in later chapters, and to highlight the potential utility of these methods for future investigations.

The redox state of secretory organelles in eukaryotic cells may be altered through the use of membrane permeable reductants and oxidants. In yeast and mammalian cells, the addition of appropriate concentrations of DTT to the growth medium selectively and reversibly inhibits the folding of secretory proteins that would normally contain structurally important disulfide bonds, such as yeast CPY and the influenza HA protein (Jämsä et al., 1994, Braakman et al., 1992). These proteins are withheld in the ER of DTT treated cells by quality control mechanisms that may eventually degrade misfolded proteins identified in the ER (Gething and Sambrook, 1992). Protein synthesis and translocation proceed normally in the presence of DTT, as does the intracellular transport of secretory proteins that lack disulfide bonds, such as the yeast periplasmic enzyme invertase (Lodish and Kong, 1993, Jämsä et al., 1994, Carlson and Botstein, 1982).

Hyperoxidizing conditions in the ER can be generated by the treatment of yeast cells with the thiol oxidant diamide (Kosower and Kosower, 1995). In this case, proteins that contain reactive thiols, such as CPY and invertase, are withheld in the ER in oxidized forms likely to contain inappropriate intra- or intermolecular disulfide bonds (Cuozzo and Kaiser, 1999). In contrast, the intracellular transport of proteins without cysteines, such as prepro-α-factor, proceeds with normal kinetics (Cuozzo and Kaiser, 1999).

Manipulation of the intracellular redox state with exogenous oxidants or reductant allows for the development of functional tests for new genes. Mutations in genes required for the transmission of oxidizing equivalents, such as yeast *ERO1*

and E. Coli dsbB, can often be suppressed by the addition of an exogenous oxidant to the growth medium. Phenotypes associated with loss-of-function mutations in these genes are exacerbated by exogenous reductants, while overexpression of these genes confers resistance to otherwise toxic levels of DTT (Frand and Kaiser, 1998, Pollard et al., 1998, Missiakas et al., 1993). In contrast, phenotypes associated with mutations in genes required for the transmission of reducing equivalents, such as yeast *GSH1*, E. coli dsbD, or E. coli dsbC, may often be suppressed by the addition of an exogenous reductant to the growth medium (Ohtake and Yabuuchi, 1991, Grant et al., 1996, Rietsch et al., 1996). Following this paradigm, clues to the function of new genes participating in redox homeostasis may be provided by assessing the sensitivity of loss-of-function and gain-of-function mutants in those genes to exogenous reductants and oxidants.

The rapid inhibition of thiol-disulfide exchange in vivo can be achieved by the treatment of yeast or bacterial cells with trichloroacetic acid (TCA), a reagent that rapidly lowers intracellular pH, thereby driving the protonation of reactive thiols. The use of acid, as opposed to thiol-alkylating reagents such as N-ethylmaleimide (NEM), provides rapid and reversible inhibition of thiol-disulfide exchange (Weissman and Kim, 1991). TCA treatment can capture mixed-disulfides between proteins undergoing thiol-disulfide exchange in vivo (Kishigami et al., 1995, Guilhot et al., 1995, Frand and Kaiser, in press).

Following the inhibition of thiol-disulfide exchange with acid, the redox state of proteins in vivo can often be assessed by the modification of free thiols with the thiol-alkylating reagent 4'-acetamido-4'-maleimidylstilbene-2,2'-disulfonic acid (AMS) at neutral pH (Kobayashi et al., 1997). When the maleimide moiety of AMS modifies a protein thiol, the apparent molecular mass of the protein increases according to the molecular mass of AMS, 0.5 kDa. The reduced and oxidized forms of proteins modified with AMS can often be resolved by SDS-PAGE.

The in vivo redox state of an oxidoreductase may provide an indication of the physiologic function of the enzyme. The active-site cysteines of oxidoreductases catalyzing disulfide bond formation, such as E. coli DsbA and DsbB, are maintained

in the oxidized form in vivo, whereas the active-site cysteines of enzymes catalyzing disulfide bond isomerization, such as DsbC and DsbD, are maintained in reduced form in vivo (Kobayashi and Ito, 1999, Kobayashi et al., 1997, Joly and Swartz, 1997, Bardwell et al., 1993, Rietsch et al., 1997, Missiakas et al., 1995).

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Chapter Two:

The *ERO1* Gene of Yeast Is Required for Oxidation of Protein Dithiols in the Endoplasmic Reticulum

PREFACE

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Summary

We have identified a conserved yeast gene, *ERO1*, that encodes a novel glycoprotein required for oxidative protein folding in the endoplasmic reticulum (ER) and is induced with the Unfolded Protein Response. A temperature-sensitive *ero1-1* mutant blocks ER-to-Golgi transport of carboxypeptidase Y (CPY), a protein with disulfide bonds, but the transport of invertase, a protein without disulfide bonds. CPY retained in the ER of an *ero1-1* mutant lacks disulfide bonds as shown by its reduced mobility on non-reducing gels and increased reactivity with the thiol-modifying reagent AMS. The level of *ERO1* gene activity appears to determine the oxidizing capacity of the cell since the *ero1-1* mutation causes hypersensitivity to the reductant DTT whereas overexpression of *ERO1* confers resistance to DTT. Moreover, the oxidant diamide can suppress both the inviability and secretion defect of *ero1* mutants. We show that the essential function of *ERO1* is distinct from that of *PDI1*, and also that glutathione is not necessary for oxidative protein folding in the ER. Together these results indicate that Ero1p is a novel and central component of the mechanism that sustains oxidizing conditions in the ER conducive to the net formation of protein disulfide bonds.

Introduction

The formation of native inter- and intramolecular disulfide bonds is a crucial step in the folding of many secretory proteins. Disulfide bonds can stabilize folded protein domains and crosslink associated subunits of oligomeric protein complexes. In eukaryotic cells, disulfide bonds form as newly translocated polypeptide chains enter the oxidizing milieu found in the lumen of the endoplasmic reticulum (ER) (Braakman et al., 1991). Experiments on

oxidative protein folding carried out both in vitro and in vivo have demonstrated that the efficient formation of native disulfide bonds requires both an oxidizing agent and a catalyst for the rearrangement of non-native disulfide bonds.

In vitro assays for oxidative protein folding typically employ a small molecule, such as oxidized glutathione (GSSG), as an electron acceptor for the oxidation of protein dithiols to disulfide bonds. GSSG can contribute disulfide bonds to the substrate or the protein catalyst through dithiol-disulfide exchange reactions (Saxena and Wetlaufer, 1970). The rate of oxidative protein refolding, regardless of the presence of a catalyst, depends on the redox potential of the assay buffer. For example, the optimum rate of PDI-catalyzed refolding of reduced ribonuclease A occurs at a [GSH]/[GSSG] ratio of 5:1 in the presence of 1 mM total glutathione (Lyles and Gilbert, 1991). Measurements of the redox conditions within the ER lumen show that the most abundant redox buffer present is glutathione and that the ratio of [GSH] / [GSSG] is about 2:1. In contrast, the ratio of [GSH] / [GSSG] in the cytosol is from 30:1 to 100:1 (Hwang et al., 1992). These findings implicate a mechanism that concentrates GSSG in the ER lumen as a potential source of oxidizing equivalents for the formation of protein disulfide bonds. However, a small molecule such as GSSG need not necessarily serve as an intermediate in protein oxidation in vivo, since disulfide bond formation in the periplasm of E. coli occurs through a series of dithiol-disulfide exchange reactions mediated entirely by periplasmic proteins (Guilhot et al., 1995; Kishigami et al., 1995).

The most extensively studied catalyst of native disulfide bond formation in the ER is protein disulfide isomerase (PDI), an abundant, 55 kD ER protein with two domains related to the active site of the dithiol-disulfide oxidoreductase thioredoxin. These domains each contain a pair of cysteine residues in the motif CXXC that can participate in dithiol-disulfide exchange reactions. In vitro, PDI

can catalyze either the formation, reshuffling, or reduction of disulfide bonds depending on the substrate and the redox conditions of the assay (reviewed by Freedman et al., 1994). The *PDI1* gene of S. cerevisiae is essential for the formation of native disulfide bonds in carboxypeptidase Y (CPY) and for cell viability (LaMantia and Lennarz, 1993). Mutational studies on the active-site cysteines in PDI indicate that the essential function of PDI in S. cerevisiae is to reshuffle non-native protein disulfide bonds (Laboissière et al., 1995).

A useful method to selectively perturb the formation of disulfide bonds in living cells is to add reduced dithiothreitol (DTT) to the growth medium. DTT can penetrate cell membranes and rapidly and reversibly inhibit protein oxidation in the ER without disrupting protein synthesis, protein translocation into the ER, or intracellular transport of proteins that do not contain disulfide bonds. For example, addition of DTT to S. cerevisiae cells blocks the folding and transport from the ER of CPY, a vacuolar protein whose native form has five disulfide bonds, but does not interfere with secretion of invertase, a protein that lacks disulfide bonds (Jämsä et al., 1994; Simons et al., 1995).

Despite the importance of the redox state of the ER for the net formation of protein disulfide bonds, little is known of how cells establish oxidizing conditions within the ER lumen. We have begun to investigate the mechanism of protein oxidation in the ER using a genetic approach. We describe a new S. cerevisiae gene, *ERO1* (ER Oxidation), mutation of which affects yeast cells in much the same way as treatment with DTT. A conditional *ero1-1* mutant is defective in the oxidation of protein dithiols in the ER and a membrane-permeable oxidant can bypass the essential function of *ERO1*. *ERO1* thus appears to define a critical component of the mechanism that sustains the oxidized redox state of the ER.

Results

Isolation of the *ERO1* Gene

In a screen of 1200 temperature-sensitive S. cerevisiae mutants (Hartwell et al., 1973) for conditional defects in export of secretory proteins from the ER, we identified a conditional allele of a novel gene that we designate *ERO1* (ER Oxidation). A backcrossed *ero1-1* strain (CKY559) failed to grow and exhibited a complete block in maturation of the ER form of CPY at temperatures above 36°C. A plasmid clone that rescued both the growth and secretion defect of *ero1-1* was isolated and the open reading frame YML130c identified as the complementing gene (Cherry et al., 1997). We verified that *ERO1* corresponds to YML130c by showing complete linkage between a *URA3* marker integrated at the YML130 locus and *ero1-1*. A chromosomal deletion of *ERO1* was constructed in a diploid strain by replacing the sequence encoding amino acids 124-500 of Ero1p with the *LEU2* marker. Sporulation of this diploid at 24°C gave only tetrads with two viable, *leu2*$^-$ spores. Thus, *ERO1* is essential for yeast viability.

ERO1 Encodes a Conserved ER Glycoprotein.

ERO1 specifies a protein with a predicted molecular weight of 65 kD; an amino terminus that appears sufficiently hydrophobic to function as a signal sequence; and 8 AsnXaaSer/Thr carbohydrate acceptor sites. Searches of Genbank failed to identify any protein of known function with sequence similarity to Ero1p. However, sequence homologs of Ero1p were found in other eukaryotic organisms including microorganisms (S. pombe and T. brucei), invertebrates (B. malayi and D. melanogaster), plants (A. thaliana), and mammals (M. musculus and H. sapiens). In the C-terminal conserved region of Ero1p, 65% of residues are identical between S. cerevisiae and humans (Figure 1A).

To monitor the intracellular distribution of Ero1p, the myc epitope was introduced at the carboxy terminus of the predicted protein coding sequence. The epitope-tagged protein was functional as *ERO1-myc* restored viability to *ero1-Δ* spores. Anti-myc recognized a 96 kD protein from cells expressing *ERO1*-myc from a centromere plasmid (CKY563). This protein was not detected from cells expressing unmodified *ERO1,* but was abundant in cells expressing *ERO1-myc* from a high copy plasmid (pAF84; Figure 1B). The apparent molecular weight of Ero1p-myc decreased to 81 kD upon treatment of cell extracts with endoglycosidase H, consistent with removal of 5-6 core N-linked oligosaccharides from Ero1p. Since Ero1p contains N-linked carbohydrate, the protein must enter the ER lumen.

To determine if Ero1p-myc was restricted to the ER or was transported to post-ER compartments, we tested for addition of α1,6-mannose to Ero1p-myc as this modification occurs in cis-Golgi. Ero1p-myc could not be re-immunoprecipitated with anti-α1,6-mannose, indicating that the majority of Ero1p-myc resides in the ER. In a control for antibody specificity, the secreted glycoprotein invertase could be re-immunoprecipitated with this antiserum (Figure 1C). Ero1p-myc was included in membrane fractions of cell extracts. The protein could be solubilized by treatment of extracts with 1% Triton X-100, but not by treatment with 0.5 M NaCl, 2.5 M urea, or 0.1 M sodium carbonate (pH 11.5). These treatments did release the luminal ER protein Kar2p from membrane fractions (data not shown). The resistance of Ero1p-myc to extraction by chaotropic agents suggested that the protein was tightly associated with ER membranes. This association may explain how Ero1p is retained in the ER without an obvious KKXX or HDEL motif for ER retention.

***ERO1* is Required for Transport of a Subset of Proteins From the ER.**

The *ero1-1* mutation inhibited transport from the ER of CPY and the GPI-linked plasma-membrane protein Gas1p, but not of the periplasmic enzyme invertase. In a pulse-chase experiment, an *ero1-1* mutant (CKY559) at restrictive temperature (38°C) exhibited a complete block in conversion of the ER form (p1) of CPY to Golgi (p2) and vacuolar (m) forms of CPY throughout 30 minutes of chase (Figure 2A). CPY could not be detected after 60 minutes of chase, suggesting that p1 CPY was eventually degraded in *ero1-1* cells (data not shown). In a wild-type strain (CKY10) under the same conditions, p1 CPY was converted to mature CPY in less than 10 minutes. Newly synthesized Gas1p is normally processed from an ER form of 120 kD to a mature, 125 kD cell-surface form (Schimmöller et al., 1995). Gas1p synthesized in an *ero1-1* mutant at 38°C remained in the ER form and comigrated during SDS-PAGE with Gas1p produced in the ER-to-Golgi vesicle formation mutant *sec12-4* (CKY39; Figure 2B). In contrast, invertase was delivered from the ER to the periplasm in less than 10 minutes in both wild-type and *ero1-1* strains at 38°C (Figure 2C). The rapid export of invertase from the ER in *ero1-1* cells indicated that the secretory pathway, including the COPII vesicles that transport both invertase and CPY from the ER, functioned in *ero1-1* cells.

An explanation for the selective secretory defect associated with *ero1-1* was based on the effect of the reductant DTT on protein transport in wild-type yeast. Addition of 5 mM DTT to wild-type cells blocks transport from the ER of CPY, but not of invertase (Jämsä et al., 1994; Figure 2A and C). Properly folded CPY contains 5 intramolecular disulfide bonds (Endrizzi, et al., 1994). CPY is retained in the ER of DTT treated cells due to a defect in the formation of these bonds (Jämsä et al., 1994). In contrast, invertase appears to lack structurally important disulfide bonds as the enzyme has only two cysteines and can fold into

an active form when expressed in the relatively reducing conditions of the cytosol (Carlson and Botstein, 1982). The maturation of Gas1p was also blocked by treatment of cells with DTT (Figure 2B), indicating that at least 2 of the 14 cysteine residues in the extracellular domain of Gas1p form a structurally important disulfide bond needed for export of Gas1p from the ER. The selective ER retention of CPY and Gas1p in the *ero1-1* mutant could thus be a consequence of a defect in oxidative protein folding.

ERO1 is Required for Oxidation of CPY.

As the *ero1-1* mutation produced a similar effect on protein transport as treatment with DTT, we postulated that the *ero1-1* mutation interfered with oxidation of protein dithiols in the ER. We therefore used non-reducing SDS-PAGE to examine the redox state of CPY in *ero1-1* cells at restrictive temperature (38°C). Indeed, CPY synthesized in an *ero1-1* mutant (CKY559) at 38°C comigrated on a non-reducing gel with reduced CPY from wild-type cells (CKY10) that had been treated with 5 mM DTT (Figure 3A). CPY produced in a *sec12-4* mutant (CKY39) that had not been treated with DTT served as a standard for oxidized, p1 CPY (Figure 3A, lane 2).

As an independent and explicit test for the presence of free thiols in CPY, we evaluated the reactivity of CPY with the thiol-conjugating reagent 4-acetamido-4'-maleimidylstilbene-2,2'-disulfonic acid (AMS). The maleimide moiety of AMS reacts with cysteine thiols, thereby increasing the molecular mass of a modified protein by approximately 0.5 kD per AMS residue added (Joly and Swartz, 1997). When *ero1-1* cells were radiolabeled at 38°C and lysed in the presence of AMS and detergent, the apparent molecular mass of CPY on a non-reducing gel increased by about 15 kD relative to that of CPY isolated in the absence of AMS (Figure 3B). This mobility shift was consistent with AMS

modification of the 10 cysteine residues normally found in intramolecular disulfide bonds in CPY. CPY synthesized in the presence of DTT reacted with AMS to the same extent. In contrast, when *sec12-4* cells were lysed in the presence of AMS, mobility of CPY increased only slightly, consistent with AMS modification of the single cysteine present in oxidized CPY (Endrizzi et al., 1994). These results show that disulfide bonds do not form in CPY expressed in an *ero1-1* mutant at restrictive temperature.

Ero1p is Induced with the Unfolded Protein Response.

Through the Unfolded Protein Response (UPR), the ER transmembrane kinase Ire1p induces expression of ER chaperones, such as *KAR2* and *PDI1*, when incorrectly folded proteins accumulate in the ER (Cox et al., 1993, Kohno et al., 1993). Ero1p-myc expression increased about 8 or 5-fold upon treatment of wild-type cells with 5 mM DTT or 10 µg/ml of the glycosylation inhibitor tunicamycin (Figure 4A), conditions known to activate the UPR. In cells overproducing Ero1p-myc from a high copy plasmid (pAF84), Ero1p-myc expression increased a further 11 or 8-fold upon treatment with DTT or tunicamycin. Cells with a chromosomal deletion of *IRE1* (CKY561) failed to induce expression of Ero1p-myc in response to these reagents and a potential Unfolded Protein Response Element (UPRE; Mori et al., 1992) was identified 401 bp upstream of the start codon of *ERO1*. The induction of Ero1p by the UPR is consistent with a role for *ERO1* in oxidative protein folding in the ER.

Partial loss of *ERO1* function appeared to produce a stress in the ER that activated the UPR. When incubated at permissive or restrictive temperature, an *ero1-1* mutant induced expression from a *UPRE-lacZ* reporter (pCF118) about 4-fold relative to a wild-type strain (Figure 4B). A similar induction of this reporter was observed when wild-type cells received 2.5 µg/ml of tunicamycin. Growth of

the *ero1-1* mutant at semi-permissive temperature may require a compensatory increase in *ero1-1* expression by the UPR since *ero1-1 ire1-Δ* double mutants were inviable at 30°C (data not shown).

Ero1p Levels Correlate with the Oxidizing Capacity of the Cell.

Otherwise isogenic strains expressing hierarchical levels of Ero1p were tested for their response to membrane-permeable reductants and oxidants. To assay the sensitivity of our strains to exogenous reductant, 10 µl of 3 M DTT were applied on sterile filter disks to lawns of cells incubated at 30°C. The growth of an *ero1-1* mutant (CKY559) was inhibited in a zone 41 ± 0.6 mm in diameter surrounding the DTT source (Figure 5A). The zone of growth inhibition for wild-type cells (CKY10) was 21 ± 0.8 mm in diameter (Figure 5A). Cells incapable of inducing *ERO1* due to deletion of *IRE1* (CKY561) were inhibited in a zone 29 ± 0.7 mm in diameter around the DTT source. The heightened sensitivity of both *ero1-1* and *ire1-Δ* mutants to DTT was also evident in growth assays of liquid cultures: 2.5 mM DTT completely inhibited growth of these mutants at 30°C but only slowed growth of a wild-type strain (Figure 5B). The extreme sensitivity of the *ero1-1* mutant to DTT at a temperature permissive for growth suggested that even a partial loss of Ero1p function greatly diminished the capacity of cells to oxidize proteins in the ER.

Increased Ero1p levels rendered cells more resistant to DTT. In the plate assay described above, the zone of growth inhibition for wild-type cells overexpressing *ERO1-myc* (from pAF84) was only 15 ± 0.7 mm in diameter around the DTT source (Figure 5A). This strain could grow to 4-times the cell density of a wild-type strain during 8 hours of growth in liquid culture containing 5 mM DTT (Figure 5B). The growth of wild-type cells in the presence of DTT was not improved by overexpression of *PDI1* or *EUG1* from the *GAL1* promoter (data

not shown). Furthermore, overexpression of *ERO1-myc* (from pAF89) in an *ire1-Δ* mutant restored to the strain wild-type resistance to DTT as observed both on plates and in liquid medium with 2.5 mM DTT (Figure 5A and B). Increased *ERO1* expression can thus account for the natural resistance of cells to DTT afforded by the UPR and Ero1p appears to be the limiting factor required for cells to overcome toxic levels of DTT.

As the *ero1-1* mutant was hypersensitive to DTT at permissive temperature, we asked whether the growth and secretion defects of this mutant at restrictive temperature could be suppressed by an exogenous oxidant. The membrane permeable diazine compound diamide drives formation of disulfide bonds in living cells (Kosower and Kosower, 1995). We applied 6 µmoles of diamide to a filter disk on a lawn of *ero1-1* cells and incubated the lawn at restrictive temperature (36°C). The *ero1-1* mutant could grow in a ring surrounding the diamide source, demonstrating that appropriate concentrations of diamide suppressed the temperature sensitivity of *ero1-1* cells (Figure 5C). Wild-type and the *ero1-1* strain displayed equal sensitivity to high diamide concentrations. By a similar plate assay, diamide was also found to rescue the inviability of *ero1-Δ* cells. A strain with a chromosomal deletion of *ERO1* covered by *ERO1-myc* on a *URA3* plasmid (CKY563) was plated on SM medium containing 5-fluoroorotic acid (5-FOA) to select for segregants that had lost the covering plasmid and 6 µmoles of diamide were applied to a filter disk on the lawn. A ring of colonies grew around the filter disk, indicating that appropriate concentrations of diamide could compensate for complete loss of *ERO1* function. These colonies were dependent on diamide for continued growth and were *ura3*$^-$, verifying that they had lost their functional allele of *ERO1*. Diamide could also restore oxidative protein folding in *ero1-1* cells. Newly synthesized CPY was rapidly transported to the vacuole in an *ero1-1* mutant growing in 0.6 mM diamide

at restrictive temperature (38°C; Figure 5C). Diamide does not appear to suppress general defects in ER protein folding since the temperature sensitivity of *kar2-159* (CKY222) and *kar2-203* (CKY229) mutants could not be rescued by the oxidant (data not shown).

Figure 5D summarizes the response of cells with different levels of *ERO1* function to exogenous reductant or oxidant. *ERO1* expression correlates with resistance to DTT and complete loss of *ERO1* function renders cells dependent on diamide. These results support the view that *ERO1* function provides the oxidizing equivalents required for oxidative protein folding in the ER and thereby sets the oxidizing capacity of the cell.

Ero1p and PDI Perform Distinct Essential Functions.

As *ERO1* appeared to encode a new type of protein required for disulfide bond formation in the ER, we explored the relationship of *ERO1* to members of the PDI gene family. The finding that exogenous diamide could compensate for loss of *ERO1* provided a useful criteria for defining gene function. We therefore tested the ability of diamide to rescue the inviability of *pdi1-Δ* cells. A strain with a chromosomal deletion of *PDI1* covered by *pGAL1-PDI1* on a *URA3* plasmid (CKY564) was plated on medium containing 5-FOA and 6 µmoles of diamide were applied to the lawn in a filter disk. Colonies were never detected on these plates (data not shown), suggesting that diamide could not provide or bypass the essential function of *PDI1*.

Functional redundancies between members of the PDI family have been revealed by observation that overexpression of either *EUG1* or *MPD1* can restore viability to *pdi1-Δ* cells (Tachibana and Stevens, 1992; Tachikawa et al., 1995). We therefore asked if *ERO1* overexpression could also rescue *pdi1-Δ* cells. A *LEU2* marked plasmid overexpressing *ERO1-myc* (pAF89) was

introduced into CKY564 and the transformants plated on medium containing 5-FOA. Colonies never appeared on these plates, indicating that Ero1p overproduction could not provide the essential function of PDI. As a positive control, we confirmed that overexpression of *EUG1* from the *GAL1* promoter (pCT44) allowed CKY564 segregants to grow in the presence of 5-FOA. In addition, overexpression of *PDI1* or *EUG1* from the *GAL1* promoter (pCT37 or pCT44) did not suppress the temperature-sensitive growth defect of the *ero1-1* mutant at 38°C (CKY559; data not shown). Together, these genetic tests indicate that Ero1p and PDI perform distinct essential functions.

Consistent with the view that Ero1p functions in the same overall pathway for native disulfide bond formation as PDI, we found the growth defect of the *ero1-1* mutant to be exacerbated by a reduction in PDI levels. Removal of the HDEL signal for ER retention from the C-terminus of PDI reduces intracellular PDI levels (LaMantia and Lennarz, 1993). In a cross between *ero1-1* (CKY559) and *PDI1-ΔHDEL* (CKY395) strains, the double mutant segregants were inviable at 24°C. This interaction appears specific since *ero1-1 KAR2-ΔHDEL* double mutants were viable at temperatures below 36°C.

Glutathione is Not Required for Oxidative Protein Folding in the ER.

As oxidized glutathione (GSSG) is an abundant disulfide bonded species in the ER (Hwang et al., 1995), we assessed the contribution of GSSG to oxidative protein folding. The *GSH1* gene of S. cerevisiae encodes γ-glutamylcysteine synthetase, the enzyme that catalyzes the first step in glutathione synthesis. Mutation of *GSH1* produces cells without detectable intracellular glutathione (Ohtake and Yabuuchi, 1991). As previously reported, we found that the presence of DTT substantially restored the ability of a *gsh1-Δ* strain (CKY565) to grow on minimal medium (Ohtake and Yabuuchi, 1991;

Grant et al., 1996; Figure 6A). This observation suggested that although GSH has a critical role as a reductant, GSSG could be dispensable as an oxidant in the ER. To test this hypothesis, the *gsh1-Δ* mutant was grown without exogenous glutathione and the processing of CPY monitored by pulse-chase analysis. In the *gsh1-Δ* mutant, p1 CPY matured to the vacuolar form at a normal rate, indicating that oxidative folding of CPY occurred in the absence of glutathione (Figure 6B). Thus, there does not appear to be an absolute requirement for GSSG as an oxidant during disulfide bond formation in the ER.

Discussion

We describe a novel gene, *ERO1*, that is required for the net formation of protein disulfide bonds in the ER. Several lines of evidence support this conclusion. (i) Ero1p is an N-linked glycoprotein restricted to the ER lumen. (ii) Ero1p expression is induced by the Unfolded Protein Response. This regulation is a hallmark of genes that assist protein folding in the ER. (iii) The conditional mutation *ero1-1* causes a complete block in the ER-to-Golgi transport of CPY, a protein whose native structure contains disulfide bonds, but does not affect the transport of invertase, a secreted protein that lacks disulfide bonds. The *ero1-1* mutation also inhibits maturation of Gas1p, a protein shown to require oxidizing conditions for export from the ER. Moreover, the *ero1-1* mutation prevents the formation of disulfide bonds in CPY as shown by an increase in the reactivity of CPY with the thiol modifying reagent AMS. (iv) A decrease in *ERO1* activity, caused by either the *ero1-1* mutation at permissive temperature or a block in *ERO1* induction imposed by deletion of *IRE1*, renders cells more sensitive to the reductant DTT. Conversely, an increase in *ERO1* activity, caused by overexpression of the gene, renders cells more resistant to DTT. (v) Although *ERO1* is required for both oxidative protein folding and for cell viability, both

processes can be restored to an *ero1-1* mutant by addition of the thiol oxidant diamide to the growth medium. These findings indicate that the essential function of Ero1p is to generate oxidizing equivalents in the ER, and further, that the oxidizing capacity of the cell correlates with the level of Ero1p activity.

Examination of the most conserved regions of Ero1p provides clues to the mechanism of *ERO1* function. At least five cysteines in Ero1p are absolutely conserved and three of these appear in the sequence CXXCXXC (see Figure 1A). This motif is known to specify part of the binding site for an iron-sulfur cluster in proteins related to ferredoxin (Beinert, 1990). This raises the intriguing possibility that the mechanism of *ERO1* function involves an electron transfer reaction at a non-heme iron center. The CXXCXXC motif could also include an active site of a thioredoxin-like oxidoreductase. Although Ero1p does not exhibit any obvious sequence similarity to proteins of the thioredoxin super-family, Ero1p may nonetheless contain a thioredoxin-like fold as primary sequence alone may not be sufficient to identify this domain (Ellis et al., 1992). Cysteine residues not included in the CXXCXXC motif could also form a reactive disulfide bond in Ero1p. We are currently evaluating the potential roles of Ero1p's conserved cysteines in the conjugation of iron and in the formation of reactive disulfide bond(s).

The essential function of Ero1p can be distinguished from that of PDI and related proteins. Whereas overexpression of genes related to *PDI1*, such as *EUG1*, can restore viability to PDI-deficient cells (Tachibana and Stevens, 1992), overexpression of *ERO1* could not. Similarly, the temperature sensitivity of an *ero1-1* mutant could not be suppressed by overexpression of *PDI* or *EUG1*. Moreover, whereas the oxidant diamide can suppress defects associated with complete loss of *ERO1* function, diamide could not restore viability to *pdi1-Δ*

cells. One model consistent with these observations is that the essential function of Ero1p in native disulfide bond formation precedes that of PDI.

We have developed an assay for the in vivo redox state of CPY based on modification of free protein thiols with AMS. This assay reveals that Ero1p is necessary for oxidation of protein dithiols in the ER. PDI is necessary for the reshuffling of non-native disulfide bonds in vivo (Laboissière et al., 1995), but the extent to which PDI may participate in protein oxidation in the ER remains unclear, in part because a direct assay for dithiol oxidation has not been performed on CPY expressed in PDI deficient cells.

As an abundant disulfide bonded species in the ER, oxidized glutathione (GSSG) has been suggested to serve as the source of oxidizing equivalents for the formation of protein disulfide bonds in the ER (Hwang et al., 1991). We therefore examined the role of glutathione in oxidative protein folding. A *gsh1-Δ* mutant, which has no detectable intracellular glutathione, carries out oxidative protein folding normally as measured by the rate of CPY maturation. Thus, GSSG does not appear to serve as an obligatory intermediate during disulfide bond formation in the ER. Since the native disulfide bonds in CPY link non-sequential cysteine residues (Endrizzi et al., 1994), glutathione also appears to be dispensable for the rearrangement of disulfide bonds. GSH and GSSG may nevertheless be important for ER function; at their high concentration, GSH and GSSG should act to buffer the redox state of the ER against transient changes.

The pathway for oxidative protein folding in the E. coli periplasm provides a useful analogy for thinking about the mechanism of oxidative folding in the ER. Disulfide bond formation in the periplasm proceeds in the absence of a small molecule oxidant such as GSSG. Disulfide bonds are donated to substrates through dithiol-disulfide exchange reactions with DsbA, a periplasmic protein structurally related to thioredoxin that contains a CXXC active site (Bardwell et

al., 1991). Oxidized DsbA is regenerated through a dithiol-disulfide exchange reaction with the cytoplasmic membrane protein DsbB (Guilhot et al., 1995; Kishigami et al., 1995). The mechanism for reoxidation of DsbB is not known. The periplasmic oxidoreductase DsbC operates in an analogous pathway for the isomerization of non-native disulfide bonds and is maintained in its dithiol form by DsbD, another dithiol-disulfide oxidoreductase found in the cytoplasmic membrane (Missiakas et al., 1995).

The properties of E. coli *dsbB* are strikingly similar to those of yeast *ERO1*. Overexpression of *dsbB* increases the resistance of E. coli cells to DTT, whereas mutation of *dsbB* renders cells more sensitive to DTT (Missiakas et al., 1993). In addition, defects in disulfide bond formation associated with null alleles of *dsbB* can be suppressed by providing cells with an exogenous source of oxidizing equivalents such as GSSG or cystine (Bardwell et al., 1993). We propose that Ero1p performs a function in the ER membrane similar to that performed by DsbB in the cytoplasmic membrane. A disulfide bond in Ero1p could ultimately be donated to a substrate protein either through a direct dithiol-disulfide exchange reaction, or through a series of such reactions mediated by the oxidoreductases related to PDI that reside in the ER lumen. In addition to *PDI1* and *MPD1*, S. cerevisiae has two ORFs, YOL088c and YIL005w, which also specify proteins that contain a thioredoxin-like motif and would be predicted to reside in the ER (Cherry et al., 1997). As a group, the five yeast proteins related to PDI could catalyze disulfide bond formation and rearrangement in the ER in much the same way as DsbA and DsbC jointly accomplish these activities in E. coli. In this respect, it is significant that mammalian PDI can partially substitute for DsbA in the periplasm of E. coli (Humphreys et al., 1995).

The mechanism by which Ero1p could be oxidized is unclear. Presumably, an oxidation-reduction process in the ER membrane could provide

the necessary oxidizing equivalents. One possibility is that oxidation of Ero1p could be coupled to one of the redox reactions of fatty acid or sterol modification that take place in the ER membrane.

With the means to isolate Ero1p from cells and examine its interactions with other proteins, it should be possible to place Ero1p in a pathway for disulfide bond formation in the ER. The apparent conservation of Ero1p suggests that the mechanism for ER oxidation in yeast is likely to be conserved in all eukaryotes.

Methods

Strains and Plasmids

S. cerevisiae strains were grown and genetically manipulated using standard techniques (Kaiser et al., 1994). YPD is rich medium with 2% glucose, YEP is rich medium with a specified carbon source, SD and SMM are minimal media (Difco Laboratories) with either 2% glucose or a specified carbon source. SMM is supplemented with 16 amino-acids not including cysteine.

Genotypes of strains used in this study appear in Table 1. The *ero1-1* mutant was isolated from a collection of temperature-sensitive (Ts^-) strains in the genetic background A364A (Hartwell et al., 1973) and backcrossed to our wild-type genetic background S288C to produce CKY558 and CKY559. In final backcrosses, temperature sensitivity and a CPY transport defect cosegregated 2:2 in all tetrads examined. pAF9 is a YCp50 library (Rose et al., 1987) plasmid that rescued growth of CKY558 at 38°C and contains a 6.5 kb genomic insert including YML130c. pAF23 carries a 2.6 kb SalI-XbaI fragment of pAF9 in pRS306 (Sikorski and Hieter, 1989). To show linkage between YML130 and *ERO1*, pAF23 linearized with EcoRI was introduced into the chromosome of CKY10 by homologous recombination. When integrants were crossed to CKY559, temperature sensitivity and uracil auxotrophy cosegregated 2:2 in all tetrads examined. *ERO1-myc* was constructed by introducing a NotI site after the last codon of *ERO1* through site-directed mutagenesis and inserting a NotI fragment encoding 3 copies of the c-myc epitope: EQKLISEEDLN. pAF82 contains *ERO1-myc* as well as genomic sequence 1156 bp 5' of the *ERO1* ATG and 394 bp 3' of the stop codon in pRS316 (*CEN URA3*). The pAF82 insert was cloned into pRS315 (*CEN LEU2*), pRS306-2μ (2μ *URA3*), and pRS305-2μ (2μ *LEU2*; Sikorski and Hieter, 1989) to create pAF85, pAF84, and pAF89, respectively.

LEU2 (from pJJ252; Jones and Prakash, 1990) was cloned into pAF23 digested with BglII and HindIII to yield pAF25. To generate CKY562, the XhoI-NotI *ero1-Δ::LEU2* fragment from pAF25 was introduced at the *ERO1* locus of a *ura3-52 leu2-3,112* diploid by one-step gene replacement. Sporulation of CKY562 transformed with pAF9 or pAF82 gave viable Ura^+ Leu^+ spore clones dependent on episomal *ERO1* for viability since Ura^- segregants could not be isolated from them on 5-fluoroorotic acid plates (5-FOA; Toronto Research Labs). CKY563 is one such clone carrying pAF82.

To construct CKY561, the *ire1-Δ::URA3* fragment from pCS109A (Cox et al., 1993) was introduced at the *IRE1* locus of CKY10 by one-step gene replacement. The *ire1-Δ* phenotype was verified by the inability of CKY561 to induce *lacZ* expression from pCF118, a *CEN LEU2* plasmid containing the 5' region of *KAR2* fused to *lacZ* (Sidrauski et al., 1996). The phenotype of the *gsh1-Δ::URA3* strain (CKY565; Schmidt et al., 1996; courtesy of Martin Brendel) was verified by plating 2 X 10^6 cells on either SD with adenine or SMM, applying either 0.05 µmoles of glutathione or 30 µmoles of DTT to the lawns in 6 mm sterile filter disks (Fisher Scientific), and incubating the lawns at 30°C for 2-5 days. On SD, CKY565 grew only in response to glutathione or DTT.

pNV31 carries *pTPI1-SUC2* in a *CEN URA3* vector (Mike Lewis). pCT37 carries *pGAL1-PDI1* in a *CEN URA3* vector; pCT44 carries *pGAL1-EUG1* in YEp351 (2µ *LEU2*; Tachibana and Stevens, 1992). pAF92 contains *pGAL1-PDI1* in pCD43 (*CEN URA3*) and was shown to overproduce PDI by immunoblotting with anti-PDI (courtesy of Peter Walter).

Radiolabeling and Immunoprecipitations

Strains were grown in SMM lacking methionine or SD with auxotrophic supplements to about 1 x 10^7 cells/ml and resuspended at 1 x 10^8 cells/ml. Cell proteins were labeled with 40 µCi of [^{35}S] methionine and cysteine (NEN-Dupont)

per OD_{600} unit of cells for 7 minutes and then chased with excess methionine and cysteine. Samples of 4 x 10^7 cells were collected in 20 mM NaN_3 and lysed by resuspension in 30 µl of 80 mM Tris-HCl pH 6.8, 2% ß-mercaptoethanol, 2% SDS, and 1 mM PMSF; boiling for 2 minutes; and agitation with glass beads. Extracts were suspended in 1ml of IP buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, I mM PMSF) and preadsorbed with fixed Staphylococcus A cells (Sigma) before incubation with primary antibody for 2 hours at 24°C. 1µl of anti-CPY, 1 µl of anti-invertase, or 4 µl of anti-Gas1p (courtesy of H. Reizman) were used per OD_{600} unit of lysate. Immune complexes were collected with Protein A Sepharose (Pharmacia), washed in IP buffer, and solubilized in 40 µl of sample buffer (80 mM Tris-HCl pH 6.8, 100 mM DTT, 2% SDS, 1 mM PMSF, 10% glycerol, 0.1% bromphenol blue). Samples were resolved by SDS-PAGE and analyzed with a 445si Phosphorimager and ImageQuant Software (Molecular Dynamics, Sunnyvale, CA).

Ts^- mutants were grown at 24°C and shifted to 38°C for 25 minutes before labeling. As indicated, cultures received 0.6 mM diamide upon shift to 38°C or 5 mM DTT 10 minutes prior to labeling. For detection of invertase, cells carrying pNV31 were labeled for 10 minutes and then converted to spheroplasts as described (Hong et al., 1996). Spheroplast pellet and supernatant fractions received CKY406 extract along with anti-invertase. Gas1p processing was monitored in a temperature-sensitive *sec6-4* mutant (CKY560) that blocks fusion of post-Golgi secretory vesicles with the plasma membrane and stabilizes mature Gas1p from proteolytic degradation.

To monitor Ero1p-myc expression, strains grown at 30°C were labeled with [^{35}S] methionine and cysteine for 30 minutes. When indicated, cells received 5 mM DTT or 10 µg/ml of tunicamycin (Sigma) 10 minutes prior to labeling. Ero1p-myc was immunoprecipitated with 1.5 µl of 9E10 (monoclonal

anti-myc) per OD_{600} unit of lysate and digested with endoH as described (Hong et al., 1996). 9E10 immune complexes were solubilized by boiling in sample buffer and divided prior to re-immunoprecipitation with either 9E10 or anti-α1,6-mannose. Cell fractionation was performed on CKY10 hosting pAF84 as described except that spheroplasts were lysed gently with a dounce homogenizer (Espenshade et al., 1995).

Assays of disulfide bond formation in CPY

To examine the mobility of CPY during non-reducing SDS-PAGE, strains were shifted from 24°C to 38°C for 20 minutes and radiolabeled as described for 30 minutes. Strains received 0 or 5 mM DTT 10 minutes before labeling. Cell samples were processed in the presence of 20 mM N-ethylmaleimide (NEM, Sigma) and lysed in the absence of ß-mercaptoethanol. CPY immune complexes were solubilized in sample buffer without DTT and resolved by SDS-PAGE. Half of each cell pellet was processed under reducing conditions (as above).

To assay the reactivity of CPY with 4-acetamido-4'-maleimidylstilbene-2,2'-disulfonic acid (AMS, Molecular Probes), cell samples derived from strains radiolabeled at 38°C for 25 minutes were divided prior to lysis in 50 µl of either 80 mM Tris-HCl pH 6.8, 1% SDS, 1 mM PMSF, and 20 mM AMS or an identical buffer without AMS. Extracts were further incubated with 100 µl of lysis buffer for 1 hour at 24°C prior to immunoprecipitation of CPY. Samples were resolved by non-reducing SDS-PAGE.

Induction of the unfolded protein response

Strains hosting pCF118 (Sidrauski et al., 1996) were grown selectively at 24°C and resuspended at 1 $X10^7$ cells/ml in YPD. Cultures were given 0 or 2.5 µg/ml of tunicamycin and incubated at 30°C or 37°C for 2.5 hours. Cells were

permeabilized and ß-galactosidase activity assayed as described (Guarente, 1983). Two transformants were assayed per strain and the experiment repeated twice.

Assays for growth in the presence of DTT or diamide

Strains were grown selectively to exponential phase at 24°C prior to assays for DTT sensitivity. Triplicate lawns of 2 x 10^6 cells were plated on YPD and 10 µl of 3 M DTT applied to the lawns in 6 mm filter disks. Diameters of the zone of inhibition were measured after incubation for 1.5 days at 30°C. Growth was also assayed by the change in OD_{600} over time of duplicate cultures given 0, 2.5, or 5.0 mM DTT and incubated at 30°C in YPD. To test for diamide rescue of Ts^- strains, lawns of 3 x 10^6 cells were plated on YPD and given 6 µmoles of diamide in a filter disk before incubation at 36°C (for CKY559) or 33°C (for CKY222 and CKY229) for 2 days. CKY563 and CKY564 (*pdi1-Δ* pCT37; courtesy of Tom Stevens) were grown overnight in 0.4 mM diamide in YPD or YEP 2% raffinose, 2% galactose. Lawns of 3 x 10^6 cells were then plated on SMM supplemented with 1 mg/ml of 5-FOA to select for cells which had lost the plasmid. The lawns received 6 µmoles of diamide on a filter disk and were incubated for 7 days at 30°C. Colonies derived from CKY563 that grew in a ring around the filter were isolated and plated surrounding a disk with 6 µmoles of diamide on SMM with or without uracil.

Tests for genetic interactions between *ERO1* and *PDI1*, *KAR2*, and *IRE1*

CKY559 transformants carrying pAF82, pCT37, pCT44, or pRS316 as well as CKY564 and CKY564 transformants carrying pCT44 or pAF89 were grown selectively in SMM 2% raffinose, 2% galactose and then resuspended at 2 X 10^7 cells/ml. CKY559 transformants were spotted on YEP 2% raffinose, 2% galactose plates and incubated at 38°C for 3 days. CKY564 transformants were

spotted on SMM with either 2% raffinose, 2% galactose or 2% glucose as well as 1 mg/ml 5-FOA and incubated at 30°C for 4-5 days.

CKY558 or CKY559 (*ero1-1*) was crossed with CKY395 (*PDI1-ΔHDEL*), CKY190 (*KAR2-ΔHDEL*), or CKY561(*ire1-Δ::URA3*). Tetrads were dissected on YPD at 24°C and double mutant progeny were identified after viable spore clones were tested for growth between 30°C and 38°C in addition to either a dominant Kar2p secretion phenotype (Elrod-Erickson and Kaiser, 1996) or uracil prototrophy. Crossing CKY558 to CKY395 gave tetrads with 4, 3, or 2 viable spores where segregation of Ts^- growth was consistent with the assignment of inviable spores as *ero1-1 PDI1-ΔHDEL* mutants.

Acknowledgments

We are especially grateful to Elizabeth Chitouras for identifying and backcrossing the *ero1-1* mutant and to Mark Winey for providing the collection of conditional mutants. We thank Peter Walter and members of his research group, Mark Rose, Tom Stevens, and Martin Brendel for strains and plasmids; and Howard Reizman for anti-Gas1p. We thank Hidde Ploegh and all members of the Kaiser lab for their advice and encouragement; Peter Chivers for critical reading of this manuscript; and John Joly and John Swartz for communicating results prior to publication.

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Figure Legends

Figure 1. *ERO1* Encodes a Conserved ER Glycoprotein.

A) The predicted amino acid sequence of Ero1p contains a potential signal sequence and eight N-linked carbohydrate acceptor sites. The four highly conserved regions shown here comprise amino acids 73-93 (1), 176-208 (2), 219-239 (3), and 332-375 (4) of Ero1p. The species of origin and accession numbers for these sequences are: Sc, Saccharomyces cerevisiae (gb|z50178); Sp, Schizosaccharomyces pombe (emb|x61926); Tb, Trypanosoma brucei (emb|x60951); Bm, Brugia malayi (gb|AA509062); Dm, Drosophila melanogaster (gb|AA202720); At, Arabidopsis thaliana (gb|T45661); Hs, Homo sapiens (gb|R07093, AA186803, R50884, AA033538).

B) Ero1p-myc migrates with an apparent molecular mass of 96 kD, and of 81 kD after endoH treatment. CKY10 (wild-type) transformants carrying either pAF9 (*CEN ERO1 URA3*; lane 1) or pAF84 (2μ *ERO1-myc URA3*; lanes 3 and 4), and CKY563 (*ero1-Δ* [p*CEN ERO1-myc URA3*]; lane 2) were labeled with [^{35}S] methionine and cysteine for 30 minutes. Ero1p-myc was immunoprecipitated from cell lysates with monoclonal anti-myc and samples treated with endoH as indicated prior to SDS-PAGE.

C) Immunoprecipitates of Ero1p-myc were re-immunoprecipitated with either anti-myc (lane 1) or anti-α1,6-mannose (lane 2). As a control, invertase was immunoprecipitated from an extract of CKY10 (wild-type) and then re-immunoprecipitated with either anti-invertase (lane 3) or anti-α1,6-mannose (lane 4). Samples were resolved by SDS-PAGE.

Figure 2. The *ero1-1* Mutant Exhibits a Defect in the Export of a Subset of Proteins from the ER.

A) CKY10 (wild-type), CKY559 (*ero1-1*), and CKY39 (*sec12-4*) were shifted from 24°C to 38°C, pulse-labeled with [^{35}S] methionine and cysteine for 7 minutes, and chased for 60 minutes. CPY was immunoprecipitated from cells collected 0, 10, 30, and 60 minutes after initiation of the chase and samples resolved by SDS-PAGE. The effect of DTT on protein transport was assessed by adding 5 mM DTT to cells 10 minutes before labeling. The p1 (ER) and mature (vacuolar) forms of CPY are indicated.

B) Gas1p was immunoprecipitated from cell lysates as in (A). To follow processing of Gas1p, a temperature-sensitive *sec6-4* mutant (CKY560) was used to prevent degradation of mature Gas1p at the cell surface. The ER (precursor) and Golgi (mature) forms of Gas1p are indicated.

C) To follow invertase transport, strains expressing invertase from the *TPI1* promoter were radiolabeled for 10 minutes and then converted to spheroplasts. Invertase was immunoprecipitated from spheroplast pellet (int) and supernatant (ex) fractions. Core-glycosylated (ER) and mature (Golgi and periplasmic) forms of invertase are indicated.

Figure 3. CPY Synthesized in an *ero1-1* Mutant Lacks Disulfide Bonds.

A) CKY10 (wild-type), CKY559 (*ero1-1*), and CKY39 (*sec12-4*) were shifted from 24°C to 38°C and then labeled with [^{35}S] methionine and cysteine for 30 minutes in the presence of 5 mM DTT (lanes 1,3, 5, and 7) or in the absence of DTT (lanes 2, 4, 6, and 8). Cells were collected and lysed under non-reducing

conditions in the presence of 20 mM NEM to block free thiols. CPY was immunoprecipitated from cell lysates and samples resolved by non-reducing (lanes 1-6) SDS-PAGE. Part of each sample was processed under reducing conditions (lanes 7-8). Reduced (red) and oxidized (ox) forms of both p1 (ER) and mature (vacuolar) CPY are indicated.

B) CKY559 (*ero1-1*) and CKY39 (*sec12-4*) were labeled at 38°C as in (A) for 25 minutes. 5 mM DTT was added to a *sec12-4* sample 10 minutes before labeling (lanes 3 and 4). Cell pellets were lysed under non-reducing conditions in the presence of 20 mM of the thiol-modifying reagent AMS (lanes 2, 4, and 6), or in the absence of AMS (lanes 1, 3, and 5). CPY was immunoprecipitated from cell lysates and samples resolved by non-reducing SDS-PAGE.

Figure 4. *ERO1* is Induced by The Unfolded Protein Response (UPR) and an *ero1-1* Mutant Activates the UPR.

A) CKY10 (wild-type) and CKY561 (*ire1-Δ*) cells hosting pAF85 (*CEN ERO1-myc LEU2*) as well as wild-type cells carrying pAF84 (2μ *ERO1-myc URA3*) were labeled with [^{35}S] methionine and cysteine for 30 minutes. 5 mM DTT or 10 μg/ml of tunicamycin (Tm) were added to cells 10 minutes before labeling as indicated. Ero1p-myc was immunoprecipitated from cell lysates and samples resolved by SDS-PAGE. Relative amounts of Ero1p-myc were quantified with a 445si Phosphorimager.

B) CKY10 (wild-type) and CKY559 (*ero1-1*) strains hosting the UPRE-*lacZ* reporter plasmid pCF118 received 0 or 2.5 μg/ml of tunicamycin before

incubation at either 30°C or 37°C for 2.5 hours. ß-galactosidase activity was assayed and normalized to 1 $0D_{600}$ unit of permeabilized cells.

Figure 5. Response of Cells With Either Increased or Decreased *ERO1* Function to Exogenous Reductant (DTT) or Oxidant (Diamide)

A) CKY10 (wild-type) with or without pAF84 (2µ *ERO1*-myc *URA3*), CKY561 (*ire1-Δ*) with or without pAF89 (2µ *ERO1-myc LEU2*), and CKY559 (*ero1-1*) were each plated as a lawn of 2 $X10^6$ cells on YPD. 10 µl of 3 M DTT were applied to each lawn in a 6 mm filter disk and the lawns incubated at 30°C for 1.5 days. The average diameter of the zone of growth inhibition for each strain was determined from 3 experiments.

B) Cultures of CKY10 (wild-type), CKY559 (*ero1-1*), and CKY561 (*ire1-Δ*) were grown to exponential phase, resuspended in YPD with 2.5 mM DTT, and incubated at 30°C. Growth of the cultures was measured as the change in optical density (OD_{600}) with time. Growth of CKY10 cells with or without pAF84 (2µ *ERO1-myc URA3*) was also monitored in the presence of 5 mM DTT.

C) CKY559 (*ero1-1*) and CKY10 (wild-type) were plated on YPD and 6 µmoles of diamide were applied to each lawn in a filter disk before incubation at restrictive temperature (36°C) for 2 days. CKY563 (*ero1-Δ* [p*CEN ERO1-myc URA3*]) was plated on SM medium supplemented with 1 mg/ml of 5-FOA to select for segregants that had lost the *URA3* plasmid. 6 µmoles of diamide were applied to the lawn in a filter disk before incubation at 30°C for 7 days. To determine the effect of diamide on CPY transport in *ero1-1* cells, CKY559 grown at 24°C was resuspended in media with 0.6 mM diamide and shifted to 38°C.

Cells were pulse-labeled with [^{35}S] methionine and cysteine for 7 minutes and chased for 30 minutes (lanes 3-5). CKY10 and CKY559 were also labeled in the absence of diamide (lanes 1 and 2). CPY was immunoprecipitated from cell lysates and samples resolved by SDS-PAGE. The p1 (ER) and mature (vacuolar) forms of CPY are indicated.

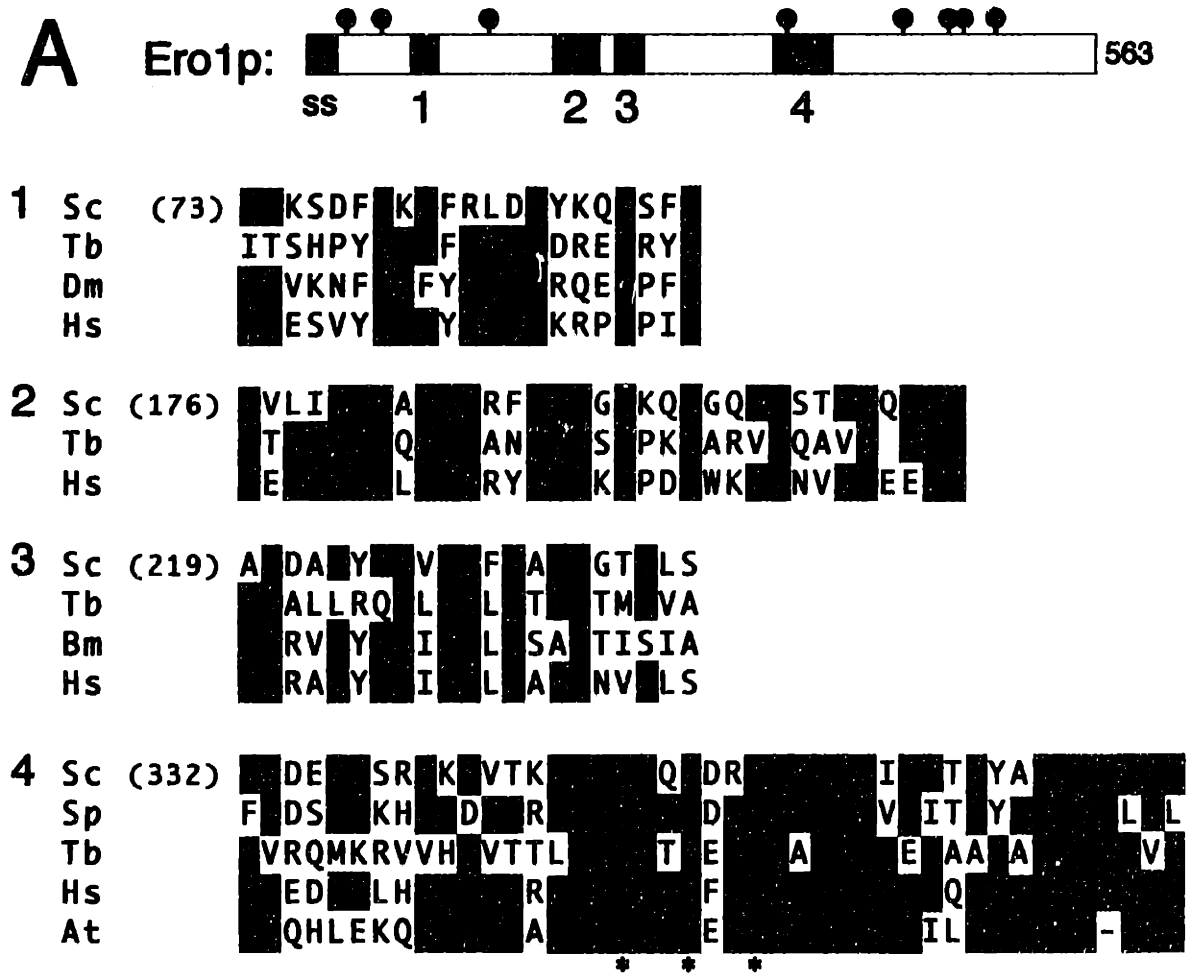
D) Diagram summarizing how the oxidizing capacity of the cell correlates with *ERO1* expression in a set of isogenic strains

Figure 6. Oxidative Protein Folding in the ER Does Not Require Glutathione.

A) Lawns of CKY565 (*gsh1-Δ::URA3*) were plated on SM medium and either 0.05 µmoles of glutathione or 30 µmoles of DTT applied to the lawns in filter disks. Plates were incubated at 30°C for 2 or 5 days, respectively.

B) CKY10 (wild-type) and CKY565 (*gsh1-Δ::URA3*) were grown in SM medium lacking methionine at 30°C, pulse-labeled with [^{35}S] methionine and cysteine for 7 minutes, and chased. CPY was immunoprecipitated from cell lysates and samples resolved by SDS-PAGE. The p1 (ER) and mature (vacuolar) forms of CPY are indicated.

Figure 1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563
ss
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2 3
4
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Hs ESVY Y KRP PI
2 Sc (176) VLI A RF G KQ GQ ST Q
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Hs E L RY K PD WK NV EE
3 Sc (219) A DA Y V F A GT LS
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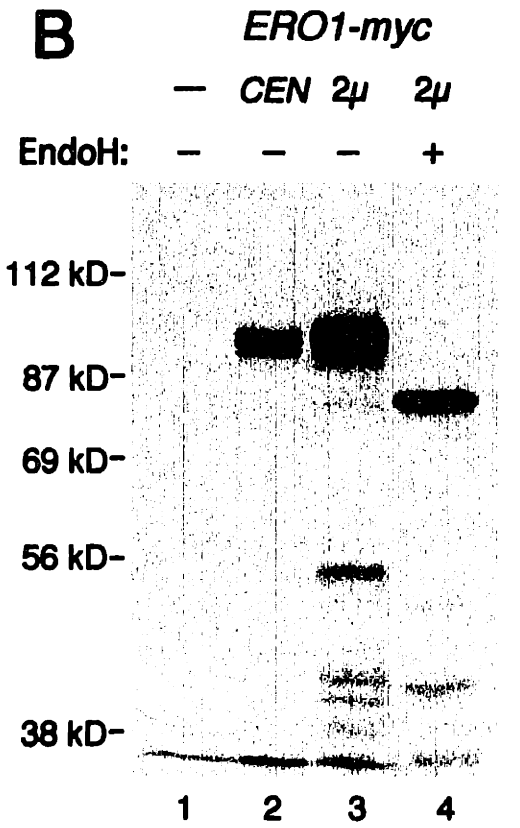
Figure 1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ERO1-myc
—
CEN
2μ
2μ
EndoH:
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–
–
+
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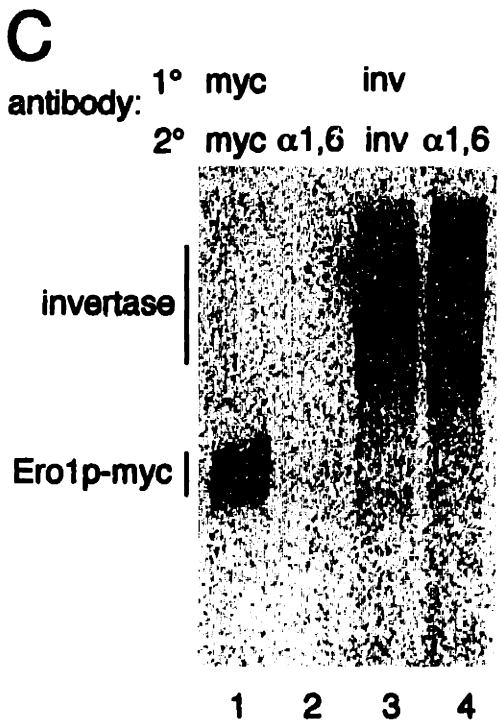
56 kD-
38 kD-
1
2
3
4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1°
myc
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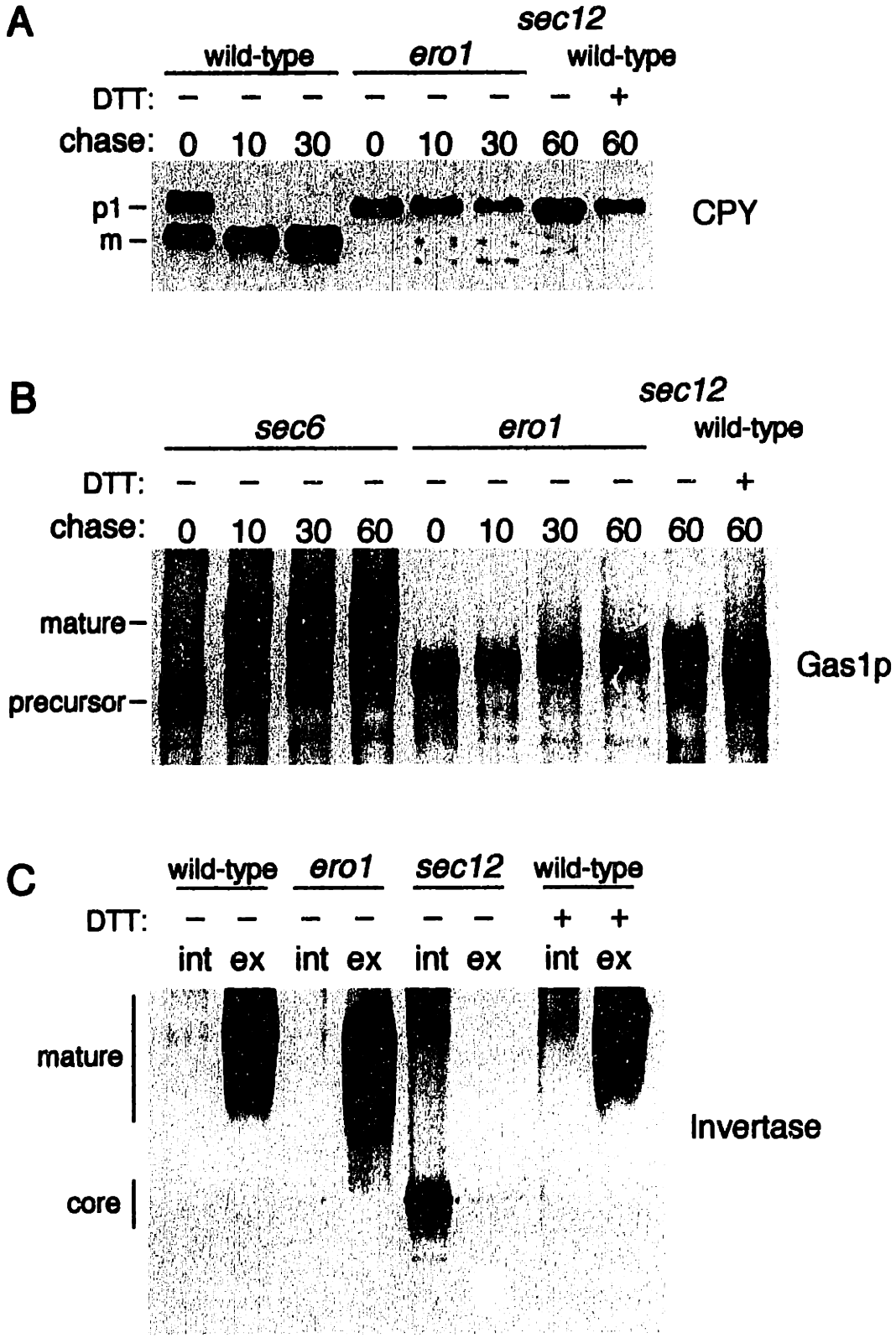
4

Figure 2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ero1
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m –
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sec12
sec6
ero1
wild-type
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precursor –
Gas1p
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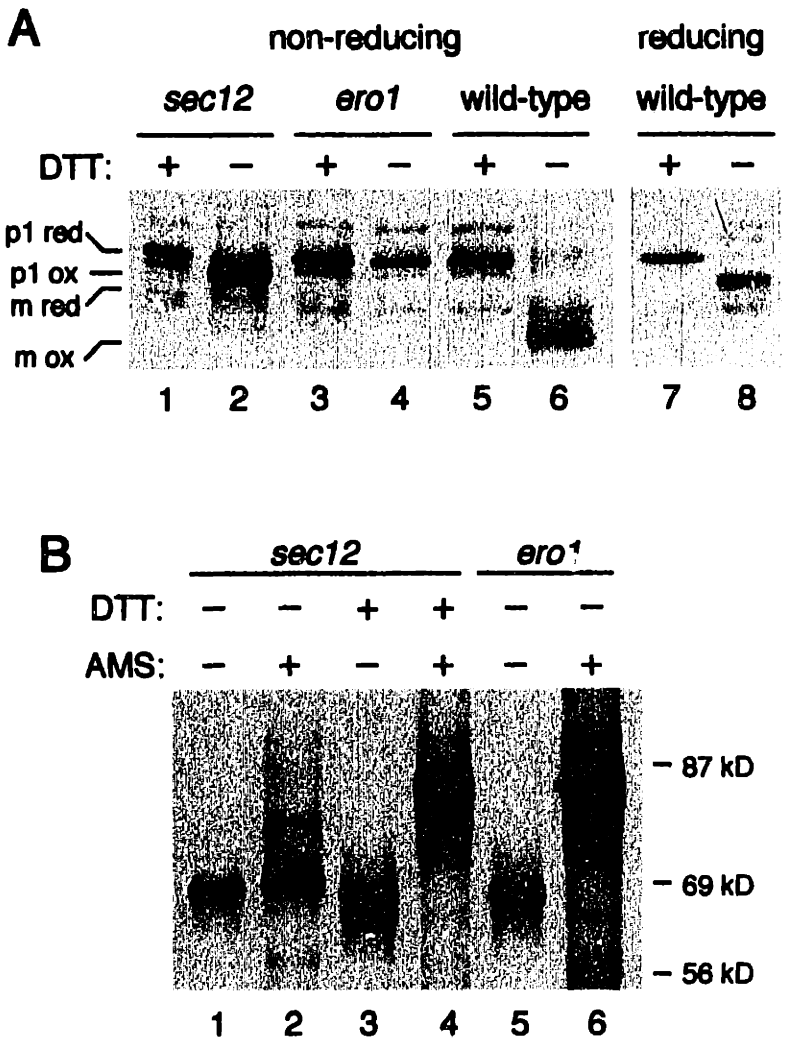
mature
core
Invertase

Figure 3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wild-type
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p1 ox
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m ox
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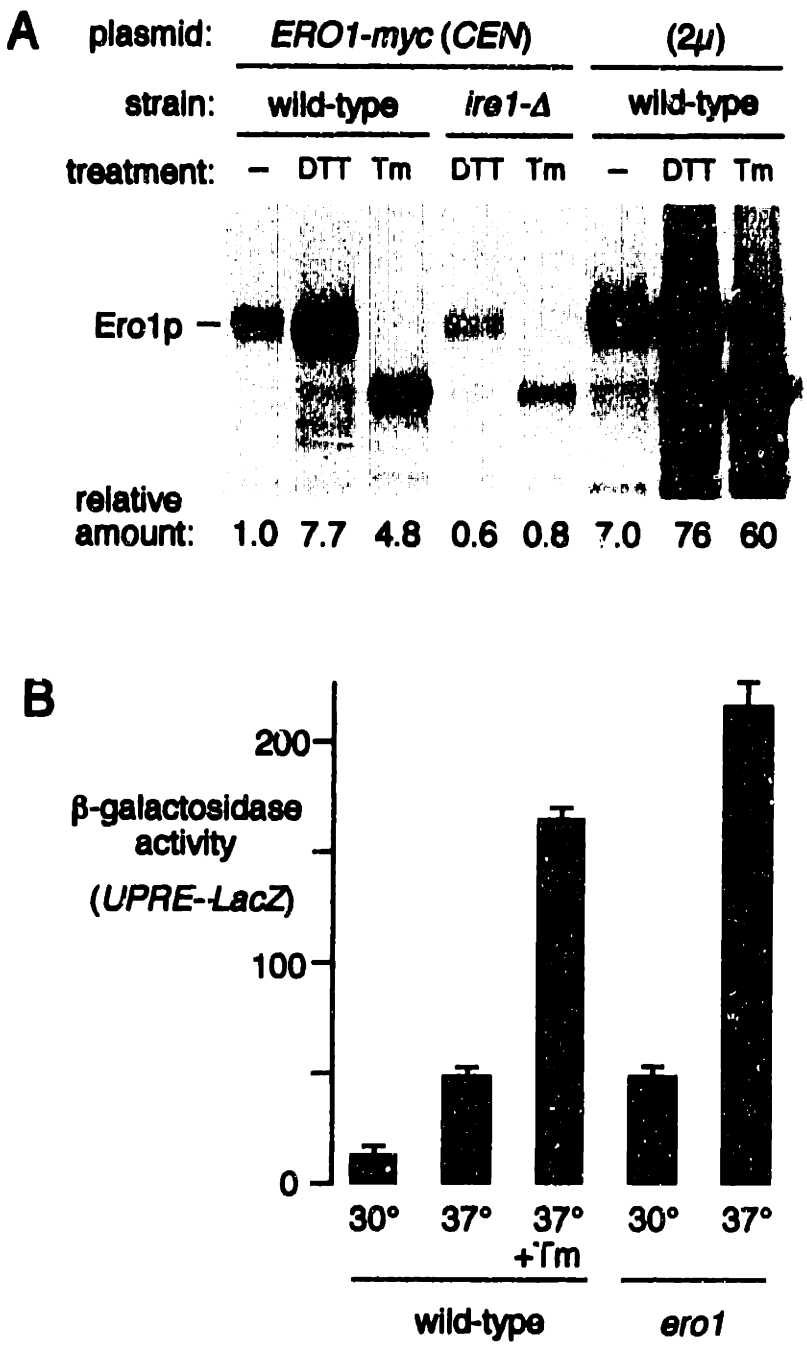
69 kD
56 kD
1 2 3 4 5 6

Figure 4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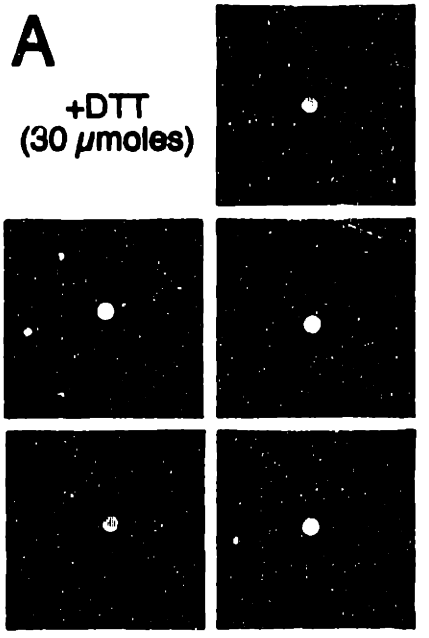
strain:
wild-type
ire1-Δ
wild-type
treatment:
– DTT Tm DTT Tm – DTT Tm
Ero1p
relative amount:
1.0 7.7 4.8 0.6 0.8 7.0 76 60
B
200
β-galactosidase activity
(UPRE-LacZ)
100
0
30° 37° 37° +Tm 30° 37°
wild-type
ero1

Figure 5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+DTT
(30 μmoles)

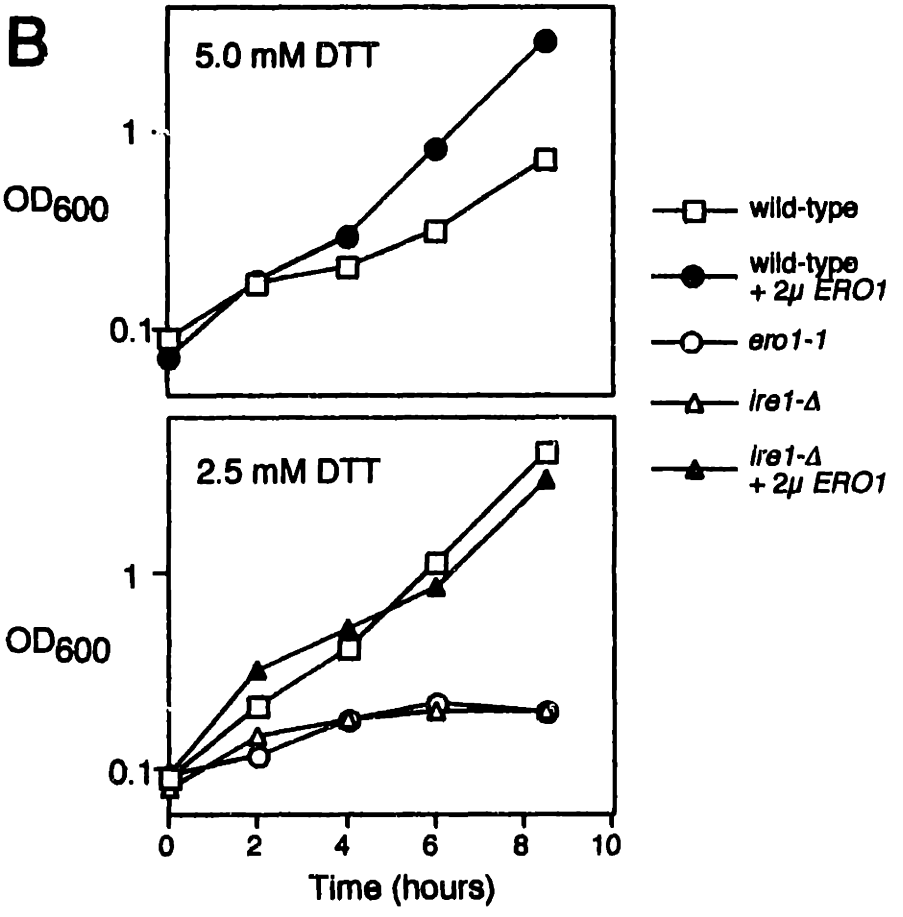
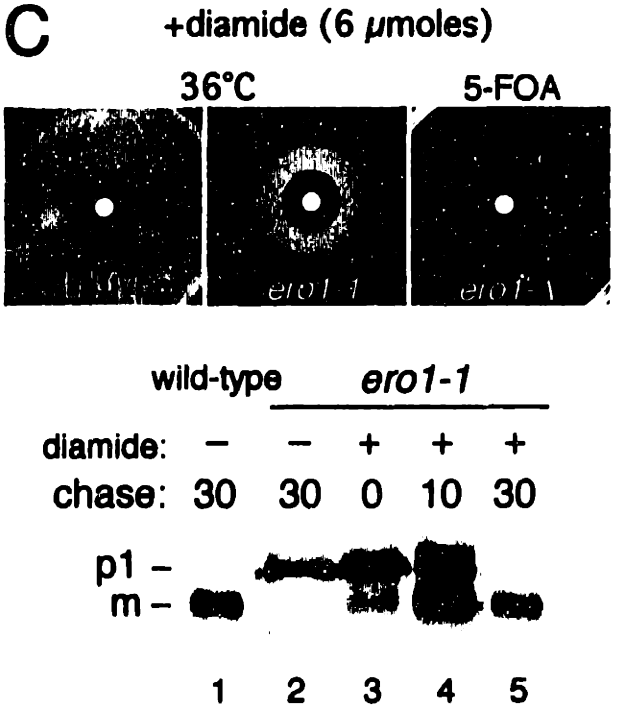

B
5.0 mM DTT
1
OD600
0.1
2.5 mM DTT
1
OD600
0.1
0
2
4
6
8
10
Time (hours)
wild-type
wild-type
+ 2μ ERO1
ero1-1
ire1-Δ
ire1-Δ
+ 2μ ERO1

Figure 5


C
+diamide (6 μmoles)
36°C
5-FOA
ero1-1
ero1-Δ
wild-type
ero1-1
diamide: – – + + +
chase: 30 30 0 10 30
p1 –
m –
1 2 3 4 5

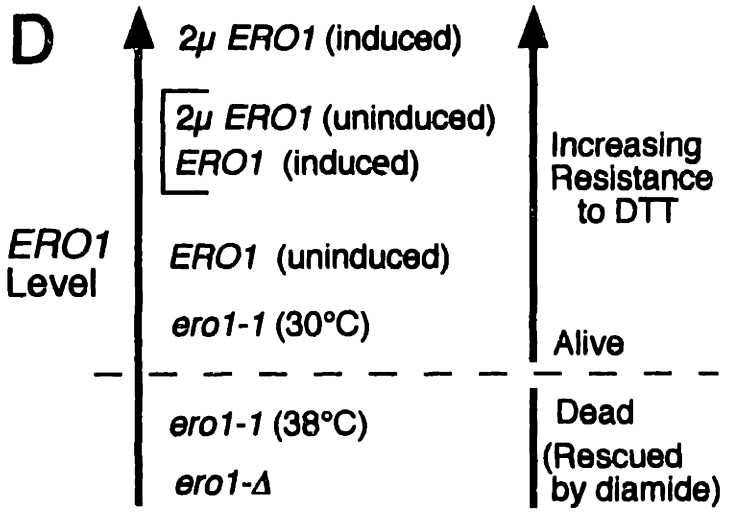

D
2μ ERO1 (induced)
2μ ERO1 (uninduced)
ERO1 (induced)
ERO1 (uninduced)
ero1-1 (30°C)
ero1-1 (38°C)
ero1-Δ
ERO1 Level
Increasing Resistance to DTT
Alive
Dead
(Rescued by diamide)

Figure 6

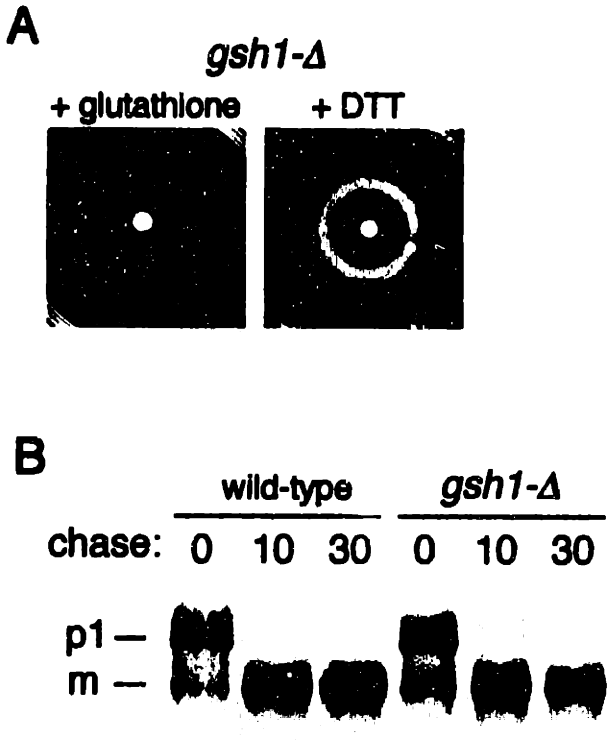

A
gsh1-Δ
+ glutathione
+ DTT
B
wild-type
gsh1-Δ
chase: 0 10 30 0 10 30
p1 —
m —

TABLE 1

Yeast Strains

Strain	Genotype	Source
CKY8	*MATα ura3-52 leu2-3,112*	Kaiser Lab Collection
CKY10	*MATa ura3-52 leu2-3,112*	"
CKY39	*MATα sec12-4 ura3-52 his4-619*	"
CKY406	*MATα suc2-Δ9 ura3-52 leu2-3,112*	"
CKY560	*MATα sec6-4 ura3-52 leu2-3,112*	This Study
CKY558	*MATa ero1-1 ura3-52 leu2-3,112 ade2*	"
CKY559	*MATα ero1-1 ura3-52 leu2-3,112*	"
CKY561	*MATa ire1-Δ::URA3 ura3-52 leu2-3,112*	"
CKY562	*MATa/α ero1-Δ::LEU2/ERO1 leu2-3,112/leu2-3,112 ura3-52/ura3-52*	"
CKY563	*MATα ero1-Δ::LEU2 ura3-52 leu2-3,112* [pAF82]	"
CKY222	*MATa kar2-159 ura3-52 leu2-3,112*	Mark Rose (MS174)
CKY229	*MATa kar2-203 ura3-52 leu2-3,112 ade2-101*	Mark Rose (MS1032)
CKY190	*MATα KAR2-ΔHDEL suc2-Δ9 ura3-52 leu2-3,112 his4-619*	Mark Rose
CKY395	*MATα pdi1-Δ::TRP1-PDI1-ΔHDEL leu2-3,112::LEU2-UPRE-lacZ*	Caroline Shamu
	ura3-1 his3-11,15 trp1-1 ade2-1 can1-100	(CS297)
CKY564	*MATα pdi1-Δ::HIS3 ura3-1 leu2-3, 112 his3-11,15 trp1-1 ade2-1 can1-100* [pCT37]	Tom Stevens
CKY565	*MATa/α gsh1-Δ1::URA3/gsh1-Δ1::URA3 leu2-Δ1/LEU2 ura3-52/ura3-52*	
	ade2-101/ade2-101 lys2-801/LYS2 trp1-Δ1/TRP1trp5/TRP5	Martin Grey (M65312)

Chapter Three:

Ero1p Oxidizes Protein Disulfide Isomerase in a Pathway for Protein Disulfide Bond Formation in the Endoplasmic Reticulum

PREFACE

This chapter will be published in *Molecular Cell* as:

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Summary

Native protein disulfide bond formation in the endoplasmic reticulum (ER) requires protein disulfide isomerase (PDI) and Ero1p. Here we show that oxidizing equivalents flow from Ero1p to substrate proteins via PDI. PDI is predominantly oxidized in wild-type cells, but is reduced in an *ero1-1* mutant. Direct dithiol-disulfide exchange between PDI and Ero1p is indicated by the capture of PDI-Ero1p mixed-disulfides. Mixed-disulfides can also be detected between PDI and the ER precursor of carboxypeptidase Y (CPY). Further, *PDI1* is required for the net formation of disulfide bonds in newly-synthesized CPY, indicating that PDI functions as an oxidase in vivo. Together, these results define a pathway for protein disulfide bond formation in the ER. The PDI-homolog Mpd2p is also oxidized by Ero1p.

Introduction

The formation of native intramolecular disulfide bonds is critical for the folding and stability of many secreted proteins. This process involves oxidation of protein thiols to form disulfide bonds as well as rearrangement of non-native disulfide bonds (Creighton, 1977, Weissman and Kim, 1991). Oxidative protein folding in vitro occurs spontaneously, but slowly, taking from hours to days (Anfinsen, 1973). In contrast, the process occurs rapidly in vivo, indicating that disulfide bond formation and rearrangement is catalyzed in living cells (Goldberger et al., 1963).

In eukaryotic cells, protein disulfide bond formation occurs in the lumen of the ER (Braakman et al., 1991). This compartment contains millimolar concentrations of reduced (GSH) and oxidized glutathione (GSSG), with the [GSH]/[GSSG] ratio ranging from 1:1 to 3:1. In the relatively reducing environment of the cytosol, the [GSH]/[GSSG] ratio ranges from 30:1 to 100:1 (Hwang et al., 1992). The oxidizing redox conditions present in the ER thermodynamically favor the formation of protein disulfide bonds. The relative abundance of GSSG in the ER has led to the proposal that GSSG serves as the source of oxidizing equivalents utilized during protein disulfide bond formation in vivo (Hwang et al., 1992). However, it has recently been

shown that glutathione is not required for oxidative protein folding in the ER (Frand and Kaiser, 1998, Cuozzo and Kaiser, 1999), indicating that disulfide bond formation in vivo relies upon a different electron acceptor.

Protein disulfide isomerase (PDI) is an abundant catalyst for native disulfide bond formation in the ER lumen. PDI contains four domains homologous to thioredoxin, two of which possess a redox-active CGHC motif (reviewed by Ferrari and Söling, 1999). In vitro, the active-site cysteines of PDI can participate in dithiol-disulfide exchange reactions catalyzing dithiol oxidation, disulfide reduction, or disulfide isomerization depending on the nature of the substrate protein and the redox conditions of the assay (reviewed by Freedman et al., 1994).

In yeast, the *PDI1* gene is essential for cell viability and for oxidative folding of the secretory marker protein carboxypeptidase Y (CPY) (Tachibana and Stevens, 1992, LaMantia and Lennarz, 1993). An important role for PDI in the isomerization of non-native disulfide bonds in vivo was revealed when an active-site mutant of PDI retaining only isomerase activity in vitro was shown to rescue the inviability of PDI-deficient cells (Laboissière et al., 1995). However, the extent to which PDI also catalyzes the net oxidation of protein thiols in vivo has remained unclear.

In addition to PDI, several other yeast ER proteins exhibit the hallmarks of a thioredoxin fold. These include Mpd1p, Mpd2p, Eug1p, and the product of ORF YIL005w (Cherry et al., 1997). Functional redundancy among these proteins has been revealed by the observation that overexpression of either *MPD1*, *MPD2*, or *EUG1* can restore growth to cells with a chromosomal deletion of *PDI1* (Tachikawa et al., 1995, Tachikawa et al., 1997, Tachibana and Stevens, 1992). Collectively, this family of proteins may catalyze both the formation and shuffling of disulfide bonds. The specific activities associated with each of these enzymes and their preferred substrates have yet to be determined.

We began a genetic dissection of oxidative protein folding in yeast with the isolation of *ERO1*, a gene encoding a novel but conserved ER membrane protein required for the net formation of protein disulfide bonds (Frand and Kaiser, 1998, Pollard et al., 1998). In a conditional *ero1-1* mutant, secretory proteins that would

normally contain intramolecular disulfide bonds, such as CPY, remain in a reduced state in the ER. Evidence that Ero1p activity introduces oxidizing equivalents into the ER lumen stems from the observations that the thiol oxidant diamide can restore CPY folding and viability in *ero1* mutants, and that overexpression of *ERO1* confers resistance to otherwise toxic levels of the reductant DTT (Frand and Kaiser, 1998, Pollard et al., 1998). These two phenotypes distinguish *ERO1* from *PDI1.* Moreover, Ero1p activity drives the production of oxidized glutathione in vivo (Cuozzo and Kaiser, 1999).

The development of several analytical techniques enabled us to assay the redox state of ER proteins and to capture intermolecular mixed-disulfides between proteins undergoing thiol-disulfide exchange in the ER. Using these methods, we have explored the functional relationship between Ero1p, PDI, and CPY. We present evidence that Ero1p directly oxidizes PDI, thereby enabling PDI to perform a critical function as a direct oxidase for newly-synthesized p1 CPY.

Results

Trapping PDI-Ero1p and Mpd2p-Ero1p Mixed-Disulfides in Vivo

The transfer of a disulfide bond between oxidoreductases involves a dithiol-disulfide exchange reaction between one enzyme in dithiol (reduced) form and a second in disulfide (oxidized) form (Figure 1A). A mixed-disulfide intermediate is generated by nucleophilic attack of the disulfide bond in the oxidized partner by a thiolate anion derived from a reactive cysteine in the reduced partner. This mixed-disulfide can be resolved by intramolecular attack of the mixed-disulfide bond by a second thiolate derived from the same enzyme (Figure 1A). Treatment of yeast cells with trichloroacetic acid (TCA) rapidly lowers intracellular pH, thereby blocking further thiol exchange and stabilizing mixed-disulfides between proteins undergoing thiol-disulfide exchange for subsequent analysis.

Since one paradigm for disulfide bond formation in the ER predicted the transfer of oxidizing equivalents from Ero1p to PDI (Frand and Kaiser, 1998, Pollard

et al., 1998), we sought to capture PDI-Ero1p mixed-disulfides in vivo. To facilitate trapping these complexes, we employed a CGHS-CGHS mutant of PDI, in which the C-terminal cysteine of each active site was replaced with serine. This mutant should be impaired in the resolution of mixed-disulfides.

Cells overproducing both Ero1p-myc and either wild-type or CGHS-CGHS PDI were labeled with [^{35}S]-methionine and then treated with TCA to rapidly inhibit further thiol exchange and precipitate cellular proteins. Free thiols were modified with the thiol-alkylating reagent N-ethylmaleimide (NEM), and the samples were adjusted to pH 6.8 before immunoprecipitation with anti-myc antibody under non-reducing but denaturing conditions. In addition to free Ero1p-myc, high molecular weight complexes could be detected (Figure 1B, lanes 2 & 3). These complexes were shown to represent PDI-Ero1p mixed disulfides by re-immunoprecipitation with anti-PDI under non-reducing conditions (Figure 1B, lane 4). The PDI-Ero1p mixed-disulfides migrated with an apparent molecular mass of approximately 230 kDa. The conformation of these complexes may have influenced their mobility during non-reducing SDS-PAGE, since this value exceeds the predicted mass of a PDI-Ero1p heterodimer. The stochiometry of these complexes may also not have been 1:1.

Primary anti-myc immunoprecipitates containing the PDI-Ero1p mixed-disulfides were reduced with DTT and divided prior to re-immunoprecipitation with anti-PDI or anti-myc antibody. The efficiency of mixed-disulfide capture could then be expressed as the ratio of PDI trapped in mixed-disulfides to the total Ero1p-myc present in each sample. By this measure, overproduced CGHS-CGHS PDI was captured in mixed-disulfides with Ero1p-myc approximately 1.4 fold more efficiently than overproduced wild-type PDI. Endogenous PDI was captured 0.35 fold as efficiently as overproduced PDI (Figure 1B, lanes 5-7). Of the total endogenous PDI, approximately 0.7% was captured in mixed-disulfides with Ero1p-myc, while as much as 1.5% of the total PDI was captured from cells overexpressing *PDI1* (data not shown). The capture of mixed-disulfides between Ero1p-myc and wild-type PDI demonstrated that complex formation was not merely a consequence of the overproduction or hyperactivity of CGHS-CGHS PDI.

Mixed-disulfides were also detected between Ero1p-myc and Mpd2p, a 31 kDa PDI homolog residing in the ER lumen (Tachikawa et al., 1997). Proteins were isolated as described above, but from cells overproducing Ero1p-myc and a CQHA active-site mutant of Mpd2p. In addition to free Ero1p-myc, a complex of 125 kDa was immunoprecipitated with anti-myc antibody under non-reducing conditions (Figure 1C, lane 3). Direct immunoprecipitation with anti-Mpd2p isolated comigrating complexes (data not shown). These complexes were not detected when the same strain was grown under conditions repressing transcription of the P_{GAL1}-*mpd2-CQHA* transgene, and were virtually undetectable in extracts from cells overproducing wild-type Mpd2p (Figure 1C, lanes 1 & 2). Further evidence that these complexes represented CQHA Mpd2p-Ero1p mixed-disulfides was derived from observation that a 35 kDa glycoprotein was released when the complexes were reduced by DTT (Figure 1C, lanes 4 & 5). Consistent with assignment of this protein as Mpd2p, a comigrating protein could be re-immunoprecipitated by anti-Mpd2p antibody following reduction of the mixed-disulfides (Figure 1C, lanes 6 & 7). In similar experiments we have thus far been unable to detect mixed-disulfides between CGHA Mpd1p and Ero1p-myc. However, this may have been a consequence of poor expression from P_{GAL1}-*mpd1p-CGHA* (data not shown), and therefore does not exclude the possibility that Ero1p also interacts with Mpd1p in vivo.

In an additional experiment, an anti-myc immunoprecipitate known to contain PDI-Ero1p mixed-disulfides was reduced and re-immunoprecipitated with anti-CPY antibody. Mixed-disulfides were not detected between CPY and Ero1p-myc (data not shown), even though CPY undergoes oxidative protein folding in the ER (Jämsä et al., 1994). This result provides an important control for the specificity of trapping intermolecular mixed-disulfides with Ero1p.

Together, these results show that PDI-Ero1p and Mpd2p-Ero1p mixed-disulfides can be trapped in vivo, indicating that Ero1p engages in thiol-disulfide exchange with these oxidoreductases in the ER.

PDI and Mpd2p are Oxidized by Ero1p.

We next examined the in vivo oxidation state of PDI and Mpd2p. To preserve the oxidation state of these proteins, thiol exchange was rapidly blocked by treatment of live cells with TCA. The oxidation state of each protein was then assayed by covalent modification with the thiol-conjugating reagent 4-acetamido-4'-maleimidylstilbene-2,2'-disulfonic acid (AMS) in the presence of denaturants (Kobayashi et al., 1997). The reduced and oxidized forms of AMS-modified proteins were resolved by SDS-PAGE.

The mobility of the vast majority of PDI isolated from wild-type cells was not altered after modification with AMS, indicating that virtually all PDI was oxidized in vivo (Figure 2A). In contrast, the apparent molecular mass of PDI isolated from *ero1-1* cells incubated at restrictive-temperature (38°C) for 1 hr increased by approximately 8 kDa upon modification with AMS. PDI that had been reduced in vivo by treatment of wild-type cells with 10 mM DTT reacted with AMS to the same extent (Figure 2A). The decreased mobility of reduced PDI after modification with AMS reflects alkylation of the four active-site cysteines and possibly of the two additional cysteines in PDI located outside the active sites. These results show that *ERO1* is required to maintain the active sites of PDI in oxidized form in vivo, and thereby reinforce the potential significance of PDI-Ero1p mixed-disulfides as physiologic intermediates in the transfer of oxidizing equivalents from Ero1p to PDI.

Although the observation that PDI is mostly oxidized in vivo suggests that PDI functions as an oxidase, a minor portion of PDI isolated from wild-type cells was modified by AMS and therefore appears to have been in the reduced form in vivo. This may reflect the role of PDI in the isomerization of non-native disulfide bonds (Laboissière et al., 1995), since disulfide shuffling requires PDI in dithiol form.

Mpd2p is predicted to contain seven cysteines, two of which comprise the active site (Tachikawa et al., 1997). After modification with AMS, the mobility of overproduced Mpd2p isolated from wild-type cells decreased slightly, indicating that Mpd2p was oxidized in vivo but contained at least one free thiol. After treatment of wild-type cells with 0.25 mM DTT, a portion of Mpd2p, which we refer to as the

singly-reduced form, was further modified by AMS, showing that Mpd2p contained at least one labile disulfide bond. To verify that the labile disulfide bond corresponded to the active site, we generated an AQHA mutant of Mpd2p by substituting alanine for both active-site cysteines. As expected, the singly reduced form of Mpd2p was not detected after DTT treatment of cells overproducing AQHA Mpd2p (Figure 2B). Treatment of wild-type cells with 1 mM or more DTT produced Mpd2p molecules that reacted even more extensively with AMS, indicating that up to two additional, likely structural, disulfide bonds were present in oxidized Mpd2p. We refer to Mpd2p molecules whose apparent molecular mass increased by 5 kDa after treatment with AMS as fully-reduced (Figure 2B). After *ero1-1* cells were incubated at 38°C for 25 min, a portion of Mpd2p was singly-reduced. After incubation of *ero1-1* cells at 38°C for 1 h, all detectable Mpd2p was either singly- or fully-reduced (Figure 2B). *ERO1* is thus required for oxidation of the active-site cysteines of Mpd2p.

PDI Functions as an Oxidase In Vivo.

Oxidized PDI could theoretically engage in thiol-disulfide exchange reactions directly oxidizing secretory proteins. We therefore sought to capture mixed-disulfides between PDI and the p1 (ER) precursor of CPY in vivo. Wild-type cells overproducing CPY were pulse-labeled with [^{35}S]-methionine for 6 min. During this time, 16% of newly-synthesized CPY remained in the ER, whereas 84% of CPY acquired carbohydrate modifications diagnostic of delivery to the cis-Golgi (p2 CPY, Figure 3A). When overproduced, the majority of CPY is secreted in this form, rather than delivered to the vacuole (Stevens et al., 1986). The labeled cells were suspended in 10% TCA and proteins were modified with NEM prior to immunoprecipitation with anti-PDI antibody under non-reducing but denaturing conditions. At this stage, discrete complexes representing mixed-disulfides between endogenous PDI and any single ER substrate were not readily detected after non-reducing SDS-PAGE. However, such complexes would be expected in extremely low abundance, since PDI is likely to interact transiently with numerous substrate proteins. Therefore, the primary anti-PDI immunoprecipitate was reduced with DTT

and re-immunoprecipitated with anti-CPY antibody. Approximately 7% of the total p1 CPY was re-immunoprecipitated under these conditions, indicating that PDI-CPY mixed-disulfides had indeed been captured (Figure 3A). The p2 (Golgi and secreted) form of CPY was not re-immunoprecipitated under these conditions, indicating that PDI-CPY mixed-disulfides were formed exclusively while CPY was folding in the ER. Of the total PDI, approximately 0.1% was captured in mixed-disulfides with p1 CPY (data not shown). This result shows that PDI participates directly in thiol-disulfide exchange with newly-synthesized CPY. These mixed-disulfides could represent physiologic intermediates in the PDI-catalyzed oxidation, reduction, or isomerization of CPY.

Previously, a requirement for PDI in the oxidative folding of CPY was established through the observation that CPY synthesized in PDI deficient cells was retained in the ER in a form that comigrated during non-reducing SDS-PAGE with reduced p1 CPY (LaMantia and Lennarz, 1993). However, since misoxidized or partially oxidized p1 CPY may co-migrate with fully reduced p1 CPY during non-reducing SDS-PAGE, this assay may not have distinguished a defect in disulfide rearrangement from a defect in disulfide bond formation. The oxidation state of p1 CPY can be assayed directly by the modification of free thiols with AMS. Modification of reduced p1 CPY with AMS increases the apparent molecular mass of the protein by 15 kDa (Frand and Kaiser, 1998), a mobility shift attributable to AMS modification of the 10 cysteines that normally form intramolecular disulfide bonds in CPY (Endrizzi et al., 1994). By this assay, newly-synthesized p1 CPY was shown to be reduced in the conditional *ero1-1* mutant (Frand and Kaiser, 1998).

The oxidation state of CPY was examined in PDI-depleted cells. A yeast strain with a chromosomal deletion of the *PDI1* gene covered by P_{GAL1}-*PDI1* (CKY564) was grown in galactose medium and then transferred to glucose medium to repress further expression of P_{GAL1}-*PDI1*. After 18 hours of growth on glucose, newly-synthesized CPY was completely retained in the ER. PDI could not be detected in extracts from these cells either by immunoprecipitation or immunoblotting with anti-PDI (data not shown, Tachibana and Stevens, 1992). The

PDI-depleted cells were radiolabeled for 10 min and treated directly with TCA to block thiol exchange in vivo and to precipitate cellular proteins. Free thiols were modified with AMS prior to immunoprecipitation of CPY. The apparent molecular mass of p1 CPY synthesized in PDI-depleted cells increased by 15 kDa upon modification with AMS (Figure 3B). Reduced p1 CPY, synthesized in the presence of 5 mM DTT, reacted with AMS to the same extent (Figure 3B). In contrast, the mobility of oxidized, native p1 CPY, synthesized in the ER-to-Golgi vesicle formation mutant *sec12*, decreased only slightly upon AMS modification (Figure 3B). These results show that CPY remained fully reduced when synthesized in the absence of PDI, indicating that PDI functions as an oxidase, not just as an isomerase, in vivo. A subset of the mixed-disulfides captured between PDI and p1 CPY may therefore represent intermediates in the oxidation of p1 CPY.

Ero1p Is Oxidized in Vivo.

We further assessed the oxidation state of Ero1p in both wild-type and PDI-depleted cells. The mobility of overproduced Ero1p-myc isolated from wild type cells was not altered by AMS modification. In contrast, after cells were treated with 5 mM DTT, modification with AMS increased the apparent molecular mass of Ero1p-myc by 16 kDa during non-reducing SDS-PAGE (Figure 4). A portion of Ero1p-myc reacted with AMS following treatment of cells with 0.5 mM DTT (Figure 4). These results show that disulfide bonds are normally formed between some pairs of the 14 cysteines in Ero1p.

In PDI-depleted cells generated as described as above, most Ero1p-myc remained oxidized (Figure 4). This result suggests that oxidizing equivalents do not normally flow from PDI to Ero1p, and thereby supports the proposal that a unidirectional flow of oxidizing equivalents from Ero1p to PDI occurs in the ER (Figure 6). The detection of small amounts of reduced Ero1p-myc in extracts from PDI-depleted cells may stem from the presence of inviable cells in this culture or from the greatly increased expression of *2μ ERO1-myc* under these conditions. The finding that Ero1p was oxidized under the same experimental conditions where p1

CPY was reduced indicates that depletion of PDI does not grossly perturb the redox state of the ER lumen.

Mpd2p Facilitates Protein Oxidation in the ER.

Evidence that Mpd2p facilitates the transfer of oxidizing equivalents from Ero1p to secretory proteins came from the isolation of a clone expressing P_{GAL1}-*MPD2* as a high-copy suppressor of *ero1-1*. This clone partially restored growth and CPY maturation in *ero1-1* cells at temperatures below 38°C (Figure 5). The suppression of *ero1-1* by P_{GAL1}-*MPD2* depended upon the oxidoreductase activity of Mpd2p, since P_{GAL1}-*mpd2-AQHA* lacked rescuing activity (Figure 5). Further, expression of P_{GAL1}-*PDI1*, P_{GAL1}-*MPD1*, or P_{GAL1}-*EUG1* was not observed to suppress *ero1-1* (data not shown), indicating that overproduced Mpd2p may serve as a relatively effective oxidase for essential secretory proteins. This difference could reflect the redox potential of Mpd2p or the expression level of P_{GAL1}-*MPD2*. The role of endogenous Mpd2p in oxidative protein folding remains to be established, in part because deletion of *MPD2* does not block maturation of CPY (data not shown).

Discussion

In S. cerevisiae, the formation of native protein disulfide bonds requires the products of two genes, *ERO1* and *PDI1* (Frand and Kaiser, 1998, Pollard et al., 1998, LaMantia and Lennarz, 1993). Here, we show that PDI-depleted cells are defective in the net formation of protein disulfide bonds in CPY, indicating that PDI acts as an oxidase in vivo. The capture of mixed-disulfides between PDI and p1 CPY indicates that PDI engages directly in thiol-disulfide exchange with newly-synthesized secretory proteins. Consistent with a role for PDI as an oxidase, the active-site cysteines of PDI are oxidized in wild-type cells. PDI becomes reduced in a conditional *ero1-1* mutant, suggesting that Ero1p is responsible for re-oxidation of PDI. Mixed-disulfides between PDI and Ero1p are also detected, consistent with the direct transfer of oxidizing equivalents from Ero1p to PDI. Ero1p itself remains

oxidized in PDI-depleted cells, suggesting that oxidizing equivalents do not flow in the reverse direction, from PDI to Ero1p. These results define a pathway for protein oxidation in the ER wherein PDI serves as an intermediate in the transfer of oxidizing equivalents from Ero1p to substrate proteins (Figure 6). Protein disulfide bond formation may proceed through this pathway without a requirement for oxidized glutathione (GSSG).

Whereas these results reveal an important role for PDI as an oxidase in vivo, most previous work has focused on isomerase activity as the cardinal function of the enzyme. The role of PDI in yeast has been studied through mutational analysis of the active-site cysteines. A CGHS-CGHS mutant of PDI, where the C-terminal cysteines of both active sites are replaced with serine, retains isomerase activity but lacks detectable oxidase or reductase activity in vitro (Walker et al., 1996, Laboissière et al., 1995). CGHS-CGHS PDI can nevertheless rescue the inviability of cells with a chromosomal deletion of *PDI1* (Laboissière et al., 1995, LaMantia and Lennarz, 1993). Overexpression of *EUG1*, which encodes a PDI homolog with CLHS and CIHS active sites, can also restore growth to PDI-deficient cells (Tachibana and Stevens, 1992). These observations indicate that the isomerase activity of PDI is essential for cell viability and have been interpreted to mean that the main function of PDI in vivo is the isomerization of non-native disulfide bonds.

Although our findings indicate that PDI has an extensive and critical role as an oxidase in vivo, isomerase activity may be limiting for growth in *pdi1Δ* cells if alternative sources of oxidizing activity can support the formation of disulfide bonds in essential secretory proteins. In the absence of PDI, Ero1p activity will still sustain oxidizing redox conditions in the ER lumen. Reduced secretory proteins withheld in the ER of PDI-deficient cells may therefore slowly become oxidized through thiol-disulfide exchange reactions with several alternative electron acceptors, including GSSG, Mpd1p, Mpd2p, or even Ero1p itself. Protein oxidation through these pathways may be relatively inefficient, and may also have to compete with the degradation of misfolded proteins. However, in PDI-deficient cells, the induction of ER oxidoreductases by the Unfolded Protein Response (Cox et al., 1993) may

facilitate protein oxidation through these pathways. Consistent with this idea, a small amount of CPY synthesized in PDI-depleted cells may become partially oxidized during prolonged labelings (data not shown).

Consistent with a role for PDI as an oxidase in vivo, *pdi1Δ* cells rescued by production of CGHS-CGHS PDI or Eug1p grow slowly and are hypersensitive to the reductant DTT (Holst et al, 1997). Complete rescue of *pdi1Δ* cells is achieved when the active sites of Eug1p are changed to redox-active CXXC motifs (Holst et al., 1997). Mutations in the internal residues of the CXXC motif of PDI can also render cells hypersensitive to DTT (Holst et al., 1997). This phenotype may stem from a deficiency in oxidase activity in vivo, since these mutations may perturb the redox potential of PDI (Holst et al., 1997, Chivers et al., 1997). Further, E. coli thioredoxin localized to the yeast ER can rescue the inviability of *pdi1Δ* cells only after the CXXC motif of the enzyme is modified to have a higher redox potential and lower p*K*a (Chivers et al., 1996, Chivers et al, 1997). These changes enable thioredoxin to serve as a more efficient isomerase, and oxidase, in vivo.

PDI was first isolated from rat liver microsomes as an activity catalyzing the re-activation of reduced, denatured RNase A (Goldberger et al., 1963). The observation that sulfhydryl oxidation was not limiting in this assay led to the suggestion that PDI was more likely to catalyze disulfide interchange than disulfide formation in vivo (Givol et al., 1964). Assays for PDI-catalysis of oxidative protein folding have since employed redox buffers containing both GSH and GSSG. The rate of oxidative folding in vitro depends upon the redox potential of the buffer, typically expressed as the $[GSH]^2/[GSSG]$ ratio at a fixed concentration of total glutathione. For example, refolding of reduced RNase A occurs most rapidly in the presence of 1.0 mM GSH and 0.2 mM GSSG (Lyles and Gilbert, 1991a). Under these conditions, PDI is generally a more effective catalyst for the rearrangement of non-native disulfide bonds than for the GSSG-dependent oxidation of protein thiols. However, this outcome may be determined by the experimental conditions, since PDI would be present in dithiol (reduced) form under the redox conditions employed and reduced PDI is suited for catalysis of disulfide shuffling. The extent to which

PDI could serve as an oxidant in vitro would be limited by the rate of oxidation of PDI by GSSG.

In a redox buffer where $[GSH]^2/[GSSG]$= 4 mM, PDI was shown to catalyze disulfide bond formation in a model peptide by accelerating the formation and resolution of peptide-glutathione mixed-disulfides, rather than by transferring disulfide bonds directly to the substrate (Darby et al., 1994). However, these redox conditions may have disfavored the direct transfer of oxidizing equivalents from PDI to the peptide by driving complete reduction of the active sites of PDI. In contrast, oxidized PDI, purified from mammalian cells, was observed to stochiometrically transfer oxidizing equivalents to a substrate protein in the absence of a redox buffer (Lyles and Gilbert, 1991b).

Like the redox buffers employed in vitro, the ER lumen contains both GSH and GSSG. Based on the relative abundance of GSSG in the ER and the activity of PDI in vitro, models for disulfide bond formation in vivo have postulated that GSSG serves as the oxidant for PDI and as the ultimate source of oxidizing equivalents for oxidative protein folding (Hwang et al., 1992). However, it has recently been shown that GSSG does not serve as an obligate intermediate in protein oxidation in vivo (Frand and Kaiser, 1998). Rather, glutathione present in the ER serves as a net reductant, with GSH becoming oxidized as a consequence of Ero1p activity (Cuozzo and Kaiser, 1999). Together, these results indicate that the transfer of oxidizing equivalents from PDI to substrate proteins is unlikely to be obligatorily coupled to re-oxidation of PDI by GSSG in vivo.

Here we present data indicating that the oxidase activity of PDI in vivo is coupled to re-oxidation of PDI by Ero1p. The finding that Ero1p activity sustains PDI predominantly in disulfide form suggests that the direct transfer of oxidizing equivalents from PDI to substrate proteins may be far more prevalent in vivo than in vitro.

The pathway for disulfide bond formation in the E. coli periplasm provides a useful analogy to the newly defined pathway for protein oxidation in the ER. In the bacterial periplasm two oxidoreductases, DsbA and DsbB, catalyze the formation of

protein disulfide bonds in the absence of small molecule intermediates. DsbA, a soluble protein, serves as the primary oxidant of substrate proteins (Bardwell et al., 1991), whereas DsbB, a cytoplasmic membrane protein, serves to re-oxidize DsbA (Bardwell et al., 1993, Missiakas et al., 1993). Mixed-disulfides between DsbA and DsbB have been detected following the treatment of bacterial cells with acid (Guilhot et al., 1995, Kishigami et al., 1995). The ability of PDI to substitute for E. coli DsbA (Humphreys et al., 1995) provides additional evidence that PDI can function as an oxidase without the involvement of GSSG.

Our results reveal a similar pathway for protein disulfide bond formation in the ER. Newly-synthesized secretory proteins can become oxidized through direct thiol-disulfide exchange reactions with oxidized PDI. PDI is then re-oxidized through dithiol-disulfide exchange with the ER membrane protein Ero1p (Figure 6). Ero1p also oxidizes the PDI homolog Mpd2p, and Mpd2p may function in parallel to PDI. The formal possibility that PDI and Mpd2p may undergo thiol-disulfide exchange with each other remains to be explored. The pathway outlined here may be highly conserved, since all eukaryotes possess homologs of PDI and Ero1p.

The isomerization of non-native disulfide bonds in ER proteins is catalyzed by PDI, possibly in addition to other PDI homologs including Eug1p. An intriguing question remains as to how the oxidase and isomerase activities of PDI may be integrated in order to expedite the formation of native protein disulfide bonds. One possibility is that GSH may sustain a portion of PDI in reduced form, thereby enabling PDI to function as an isomerase (Cuozzo and Kaiser, 1999).

In our model, the flux of disulfide bonds into the ER lumen depends upon oxidation of redox-active cysteines in Ero1p. The mechanism responsible for oxidizing Ero1p is unclear. However, the tight association of Ero1p with the ER membrane (Frand and Kaiser, 1998) may allow oxidation of Ero1p to be coupled to electron-transport reactions mediating fatty-acid and sterol modification in the ER membrane. Given the similarities between Ero1p and DsbB, the recent finding that oxidation of DsbB is coupled to respiratory chain function in the cytoplasmic membrane (Kobayashi and Ito, 1999) may prove useful in analyzing Ero1p.

Experimental Procedures

S. cerevisiae strains were grown and genetically manipulated using standard techniques (Kaiser et al., 1994). Table 1 describes strains employed in this study. YPD is rich medium with 2% glucose; YEP Raf/Gal contains 2% raffinose and 2% galactose. SMM is minimal medium supplemented with amino acids and 2% glucose; SMM Raf/Gal contains 2% raffinose and 2% galactose. One OD_{600} U corresponds to 2 $x10^7$ cells. Cells were labeled with [^{35}S]-methionine and cysteine [EXPRESS, NEN], and kinetic analyses of CPY transport were performed as described (Frand and Kaiser, 1998).

Plasmids

To generate pAF132, a 1.8 Kb BamHI-NotI fragment corresponding to *pdi1-CGHS-CGHS* was isolated from pRH1966 (Holst et al., 1997) and placed under *GAL1* promoter expression through homologous recombination in vivo with MscI and AatII digested pCT37 (*CEN* P_{Gal1}*-PDI1 URA3*; Tachibana and Stevens, 1992). pAF103 (*CEN* P_{Gal1}*-MPD2 URA3*) was isolated from a yeast P_{GAL1}- cDNA library (Liu and Bretscher, 1992) as a suppressor of the growth defect of CKY559 (*ero1-1*) at 36°C. pAF123 (P_{GAL1}*-mpd2-CQHA*) and pAF150 (P_{GAL1}*-mpd2-AQHA*) were generated by site-directed mutagenesis on pAF103. Plasmid pTSY3 specifies *2μ PRC1 URA3* (Stevens et al., 1986).

Trapping Mixed-Disulfides

Radiolabeled cells were harvested by centrifugation and suspended in 100 μl of 10% (w/v) trichloroacetic acid (TCA). Cell membranes were disrupted by agitation with glass beads and proteins collected by centrifugation at 4°C. Protein pellets were washed with 1 ml of acetone and solubilized in 34 μl of non-reducing sample buffer (80 mM Tris-HCl, 2% SDS, 1 mM PMSF, Bromophenol Blue) containing 40 mM N-ethylmaleimide (NEM, Sigma). In experiments requiring samples larger than 4 OD_{600} U, multiple protein pellets were used, each corresponding to 5 OD_{600} U solubilized in 50 μl of buffer. Protease inhibitors (1μg/ml each pepstatin, aprotinin,

and leupeptin) were included when trapping PDI. Samples were adjusted to pH 6.8 by gradual addition of 1M Tris-HCl pH 6.8. After incubation on ice for 15 min, and at room temperature for 10 min, samples were brought to 1 ml in IP buffer and immunoprecipitated as described (Frand and Kaiser, 1998). For capture of PDI-Ero1p-myc mixed-disulfides, cells were radiolabeled in SC Raf/Gal with appropriate auxotrophic supplements and lacking methionine for 30 min at 30°C. Extracts corresponding to 15 OD_{600} U were immunoprecipitated with anti-myc antibody (9E10, Covance). One sample from cells hosting pAF132 was re-immunoprecipitated with anti-PDI under non-reducing conditions. Otherwise, 1/5 of each sample was saved for analysis by non-reducing SDS-PAGE, and the rest boiled for 3 min in sample buffer with 100 mM DTT. 1/5 of each sample was re-immunoprecipitated with 9E10 antibody while 3/5 was re-immunoprecipitated with anti-PDI antibody (kindly provided by Tom Stevens). As a control, supernatants from this last step were immunoprecipitated with anti-CPY antibody. To quantify free PDI, PDI was immunoprecipitated from the 1° anti-myc supernatants under reducing conditions. Following SDS-PAGE, proteins were visualized with a 445si phosphorimager and quantified with ImageQuant software (Molecular Dynamics). The efficiency of mixed-disulfide capture is expressed as the ratio of the band intensity of re-immunoprecipitated PDI (per OD_{600} U of extract) to that of re-immunoprecipitated Ero1p-myc (per OD_{600} U of extract). When appropriate, the value corresponding to endogenous PDI was subtracted from the numerator. Each measurement was normalized to the value obtained with overproduced, wild-type PDI. Samples with CQHA Mpd2p were prepared similarly, but the 2° IP was performed with anti-Mpd2p antibody (1.5 µl per OD_{600} U; kindly provided by Hiroyuki Tachikawa). The molecular weight of CQHA Mpd2p-Ero1p-myc mixed-disulfides was assessed by 6% SDS-PAGE. For trapping PDI-CPY mixed-disulfides, wild-type cells hosting pTSY3 were radiolabeled for 6 min. An extract corresponding to 35 OD_{600} U was immunoprecipitated with anti-PDI (1.5 µl per OD_{600} U) under non-reducing conditions. A portion of the sample was saved for analysis by non-reducing SDS-PAGE, and the rest boiled for 3 min in sample buffer with 100 mM DTT. Sample

corresponding to 28 OD_{600} U of extract was re-immunoprecipitated with anti-CPY antibody (kindly provided by Hidde Ploegh). To quantify free CPY, the supernatant from the anti-PDI IP was immunoprecipitated with anti-CPY antibody under reducing conditions.

Determination of the Oxidation State of PDI, Mpd2p, and Ero1p

For analysis of Mpd2p, cells hosting pAF103 or pAF150 were grown to exponential phase in SMM Raf/Gal lacking uracil and resuspended at 5 OD_{600} U/ml. For Ero1p-myc, cells hosting pAF89 (Frand and Kaiser, 1998) were grown in SMM lacking uracil and resuspended at 3 OD_{600} U/ml. For PDI, cells grown in YPD were resuspended at 3 OD_{600} U/ml in SMM. Samples receiving DTT (in the amounts indicated) were incubated at 30°C for 45 or 30 (for Ero1p-myc) min. *ero1-1* strains were incubated at 38°C, and were returned to 38°C for 8 min after harvesting. Cells were collected by centrifugation and suspended in 100 µl of 10% TCA. Proteins were collected as described and solubilized in non-reducing sample buffer with or without 25 mM 4-acetamido-4'-maleimidylstilbene-2,2'-disulfonic acid (AMS, Molecular Probes). For analysis of PDI, this buffer contained 6 M urea. Samples were incubated on ice for 15 min, at 37°C for 10 min (PDI and Ero1p-myc only), and boiled for 2 min. Ero1p-myc samples were de-glycosylated by 4-fold dilution into 50 mM sodium citrate pH 5.5 containing 100 U of $EndoH_f$ and incubation at 37°C for 2 h. Samples were resolved by non-reducing (PDI and Ero1p-myc) or reducing (Mpd2p) SDS-PAGE. Western analysis was performed as described (Elrod-Erickson and Kaiser, 1996) with anti-PDI (1:1,000 dilution), anti-Mpd2 (1:5,000 dilution), or 9E10 (1:1,000 dilution) as 1° antibody, and donkey anti-rabbit IgG-HRP (1:10,000 dilution) or sheep anti-mouse IgG-HRP (1:10,000 dilution, Amersham) as 2° antibody. Westerns were developed by chemiluminescence (ECL system, Amersham).

Determination of the Oxidation State of CPY

CKY564 (*pdi1Δ* pCT37, Tachibana and Stevens, 1992) was grown to exponential phase in YEP Raf/Gal and diluted over 200-fold into SMM lacking methionine and grown in exponential phase for 18 hours. CKY564 and CKY39 (*sec12-1*) were then radiolabeled for 10 min at 30°C or 38°C, respectively. One CKY39 sample received 5 mM DTT prior to labeling. Cells were harvested by centrifugation and suspended in 100 µl of 10% TCA. Proteins were collected as described and solubilized in 34 µl of non-reducing sample buffer with or without 25 mM AMS. Samples were brought to pH 6.8, incubated on ice for 15 min, at room temperature for 5 min, and boiled for 2 min. Samples were immunoprecipitated with anti-CPY antibody as described (Frand and Kaiser, 1998). Samples were resolved by SDS-PAGE and visualized with a 445si phosphorimager (Molecular Dynamics).

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Figure Legends

Figure 1 Trapping PDI-Ero1p and Mpd2p-Ero1p Mixed-Disulfides.

A) Proposed mechanism of thiol-disulfide exchange between Ero1p and a thioredoxin-like domain of PDI. The mixed-disulfide intermediate is stabilized by acid due to protonation of reactive thiols, and also by replacement of each C-terminal active-site cysteine of PDI with serine.

B) Radiolabeled cells overproducing Ero1p-myc in addition to wild-type (lanes 2 & 6) or CGHS-CGHS PDI (lanes 3 & 7) were suspended in 10% TCA to block thiol exchange in vivo. Free thiols were modified with NEM (pH 6.8) prior to immunoprecipitation with anti-myc antibody under non-reducing conditions. In addition to free Ero1p-myc, 230 kDa complexes were detected (lanes 2 & 3) that were re-immunoprecipitated with anti-PDI antibody under non-reducing conditions (lane 4, 5x loading). The anti-myc immunoprecipitates were reduced with DTT, divided, and re-immunoprecipitated with anti-PDI or anti-myc antibody (lanes 5-7), allowing quantification of the PDI trapped in mixed-disulfides with Ero1p-myc (see Methods). Anti-PDI samples were loaded 6x relative to anti-myc samples. Note that yeast PDI often migrates as a doublet. Strains were derived from CKY263 (wild-type *GAL2*) and hosted pAF89 (*2μ ERO1-myc*, lanes 1 & 5) in addition to pCT37 (P_{GAL1}-*PDI1*, lanes 2 & 6) or pAF132 (P_{GAL1}-*pdi1-CGHS-CGHS*, lanes 3, 4 & 7).

C) Primary anti-myc immunoprecipitates were prepared as in part B from labeled cells overproducing either wild-type or CQHA Mpd2p in addition to Ero1p-myc. CQHA Mpd2p-Ero1p mixed-disulfides are indicated (125 kDa, lane 3). Reduction of these complexes with DTT released a protein of 35 kDa whose mobility increased after de-glycosylation with EndoH_f (lanes 4 & 5). In a parallel experiment, this 35 kDa protein (lane 7) was re-immunoprecipitated by anti-Mpd2p antibody (lane 6, 3x

loading). Strains were derived from CKY263 and hosted pAF89 in addition to pAF123 (P_{GAL1}-*mpd2-CQHA*, lanes 1 & 3-7) or pAF103 (P_{GAL1}-*MPD2*, lane 2). In lane 1, expression of P_{GAL1}-*mpd2-CQHA* was repressed by growth on glucose.

Figure 2 *ERO1* maintains PDI and Mpd2 in Oxidized Form In Vivo.

A) Proteins were precipitated with TCA from *ero1-1* cells (CKY559) incubated at 38°C for 60 min, and from wild-type cells (CKY10) incubated in the presence or absence of 10 mM DTT at 30°C. Free protein thiols were modified with AMS in the presence of 6 M urea and 2% SDS, and samples resolved by SDS-PAGE. PDI was detected by immunoblotting. The reduced (red) and oxidized (ox) forms of PDI are indicated.

B) *ero1-1* cells (CKY598) expressing P_{GAL1}-*MPD2* were incubated at 38°C for 25 or 60 min, and wild-type cells (CKY263) expressing P_{GAL1}-*MPD2* or P_{GAL1}-*mpd2-AQHA* were incubated in the absence or presence of DTT, as indicated. Proteins precipitated by TCA were modified with AMS in 2% SDS. Mpd2p was detected by immunoblotting. The oxidized (ox), singly- (red1), and fully-reduced (red) forms of Mpd2p are indicated.

Figure 3 PDI Functions as an Oxidase *in vivo*.

A) Detection of mixed-disulfides between PDI and newly-synthesized CPY. Wild-type cells overproducing CPY were labeled with [^{35}S]-methionine for 6 min prior to suspension in 10% TCA. Free thiols were modified with NEM prior to immunoprecipitation with anti-PDI antibody under non-reducing but denaturing conditions. Reduction of the sample with DTT followed by re-immunoprecipitation with anti-CPY antibody isolated p1 CPY captured in mixed-disulfides with PDI. A 1° anti-CPY immunoprecipitate was prepared from the same cells. The p1 (ER) and p2 (Golgi and secreted) forms of CPY are indicated.

B) *PDI1* is required for efficient oxidation of CPY. To deplete cells of PDI, a *pdi1Δ* strain expressing P_{GAL1}-*PDI1* (CKY564) was grown in 2% glucose for 18 hours. Cells were then radiolabeled with [^{35}S]-methionine for 10 min. Proteins were TCA-precipitated and solubilized in the presence or absence of 25 mM AMS prior to immunoprecipitation of CPY. To provide standards for the reactivity of reduced and oxidized p1 CPY with AMS, a *sec12-4* strain (CKY39) was labeled at 38°C in the presence or absence of 5 mM DTT.

Figure 4 Ero1p is Oxidized in vivo.

Wild-type (CKY263) and PDI-depleted cells hosting pAF89 (*2μ ERO1-myc*) were incubated in the presence or absence or DTT as indicated. TCA-precipitated proteins were solubilized in the presence or absence of 25 mM AMS and de-glycosylated with EndoH$_f$. Ero1p-myc was detected by immunoblotting. The oxidized (ox) and reduced (red) forms of Ero1p are indicated.

Figure 5 *MPD2* Facilitates Oxidative Protein Folding in the ER.

P_{GAL1}-*MPD2* partially restores growth and oxidative protein folding in the *ero1-1* (CKY598) mutant, whereas the active-site mutant P_{GAL1}-*mpd2-AQHA* does not.
A) Strains were grown selectively to exponential phase, plated on YEP Raf/Gal at a density of 4x10^7 cells/ml, and incubated at 36°C for 2 days. CKY598 transformed with vector (pRS316) is also shown.

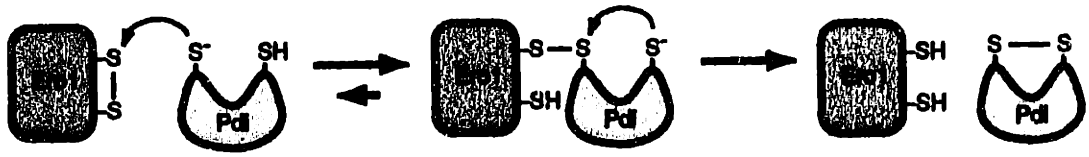
B) Cells growing in SMM Raf/Gal were pulse-labeled with [^{35}S]-methionine for 7 min at 37°C, and then chased with excess methionine and cysteine. CPY was immunoprecipitated and the samples resolved by SDS-PAGE. The p1 (ER) form of CPY and the mature (vacuolar) form of CPY, synthesized in wild-type cells (CKY263), are indicated.

Figure 6 Model for Protein Disulfide Bond Formation in the ER Lumen

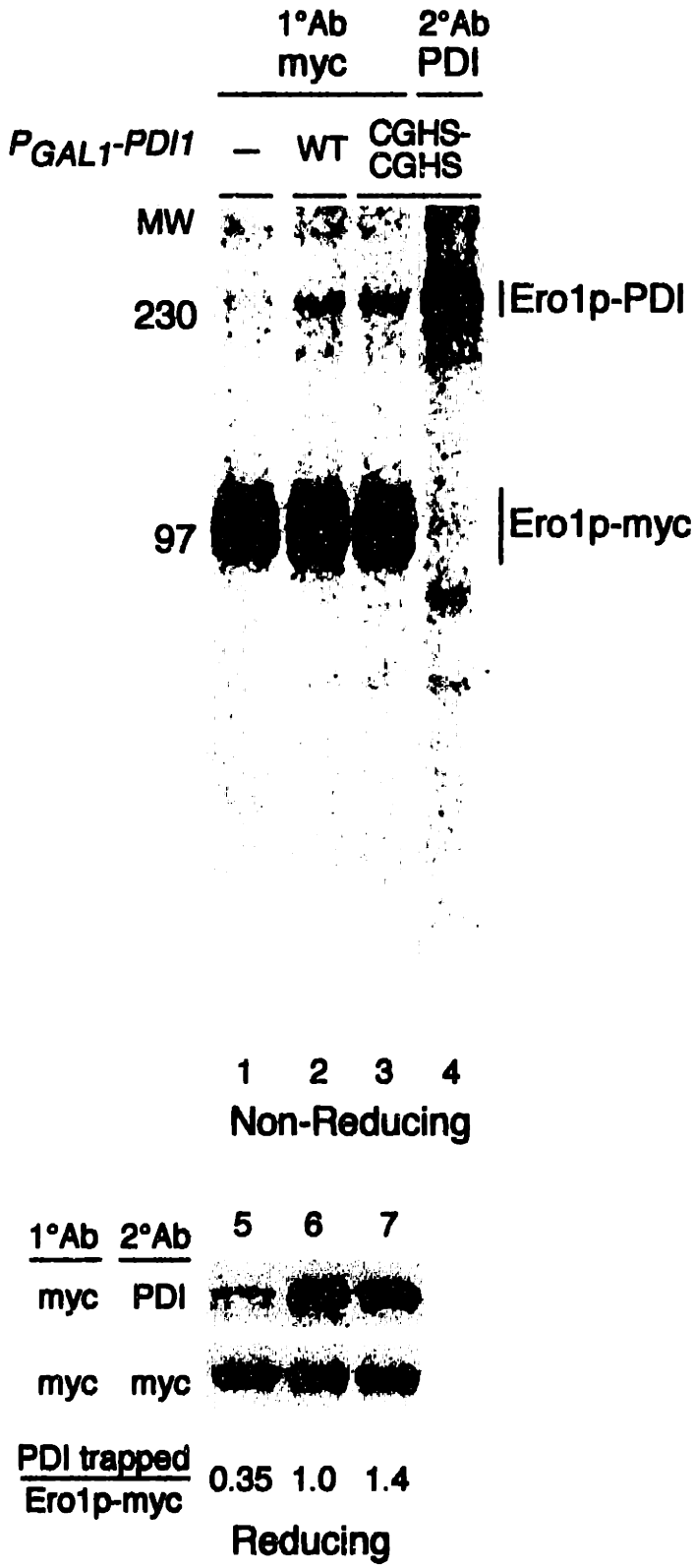
PDI is oxidized through dithiol-disulfide exchange with Ero1p. Proteins folding in the ER can then be oxidized through thiol-disulfide exchange with PDI. Only one active site of PDI is shown. The PDI homolog Mpd2p is also oxidized by Ero1p and may function in parallel to PDI.

Figure 1

A)



B)


1°Ab
myc
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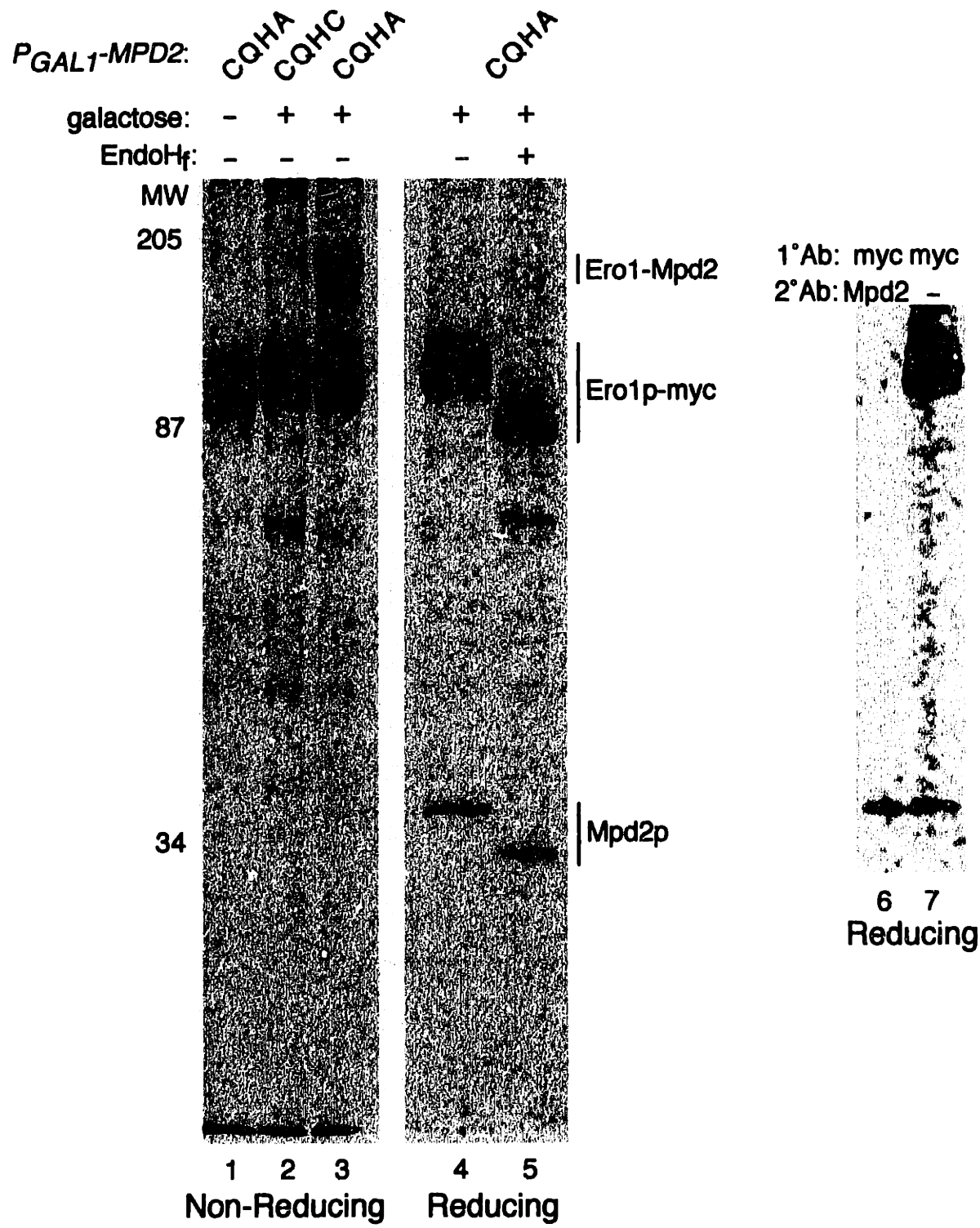
PDI
P_{GAL1}-PDI1
—
WT
CGHS-CGHS
MW
230
97
Ero1p-PDI
Ero1p-myc
1 2 3 4
Non-Reducing
1°Ab 2°Ab
5 6 7
myc PDI
myc myc
PDI trapped / Ero1p-myc
0.35 1.0 1.4
Reducing

C)


P_{GAL1}-MPD2:
CQHA
CQHC
CQHA
CQHA
galactose: - + + + +
EndoH$_f$: - - - - +
MW
205
87
34
Ero1-Mpd2
Ero1p-myc
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1 2 3
Non-Reducing
4 5
Reducing
1°Ab: myc myc
2°Ab: Mpd2 -
6 7
Reducing

Figure 2

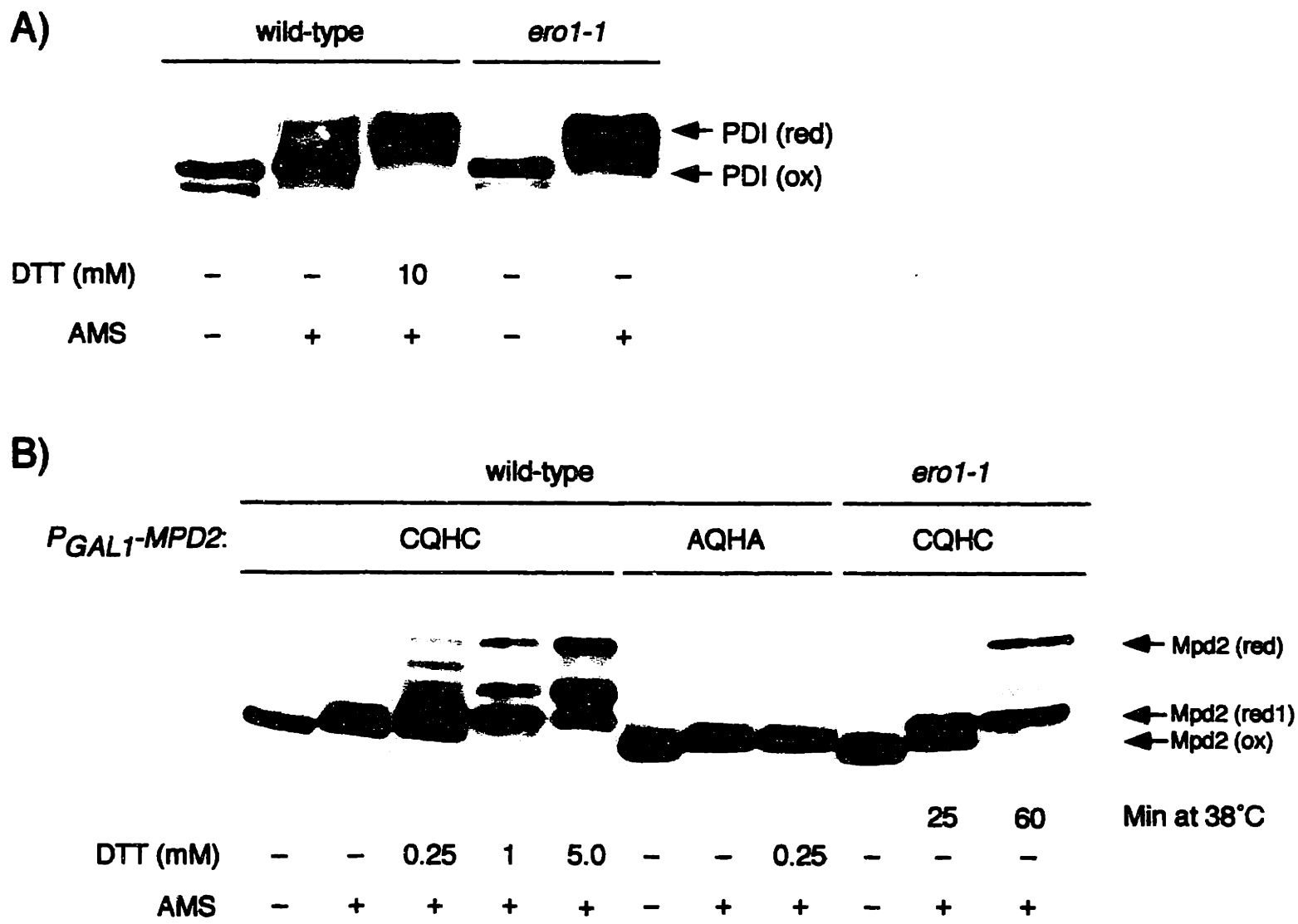
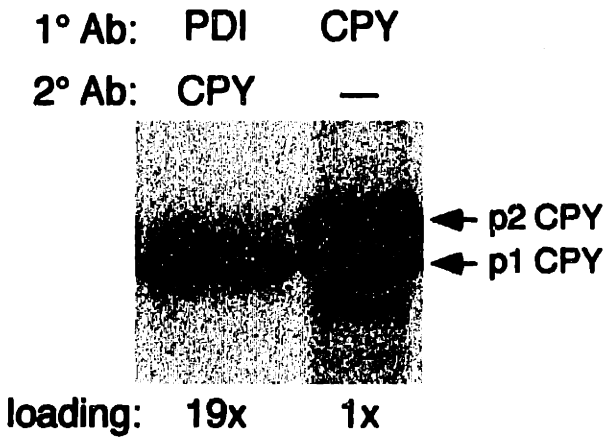

A)
wild-type
ero1-1
PDI (red)
PDI (ox)
DTT (mM) – – 10 – –
AMS – + + – +
B)
wild-type
ero1-1
PGAL1-MPD2:
CQHC
AQHA
CQHC
Mpd2 (red)
Mpd2 (red1)
Mpd2 (ox)
25 60
Min at 38°C
DTT (mM) – – 0.25 1 5.0 – – 0.25 – – –
AMS – + + + + – + + – + +

Figure 3

A)


1° Ab: PDI CPY
2° Ab: CPY —
p2 CPY
p1 CPY
loading: 19x 1x

B)

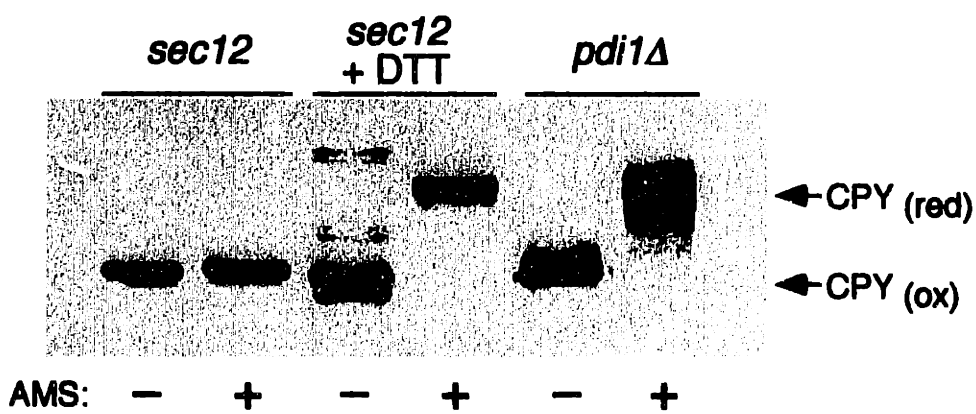

sec12
sec12 + DTT
pdi1Δ
CPY (red)
CPY (ox)
AMS: − + − + − +

Figure 4

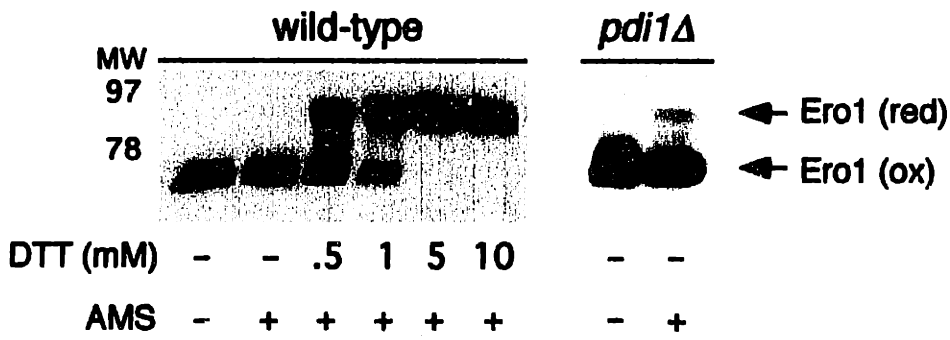


Figure 5

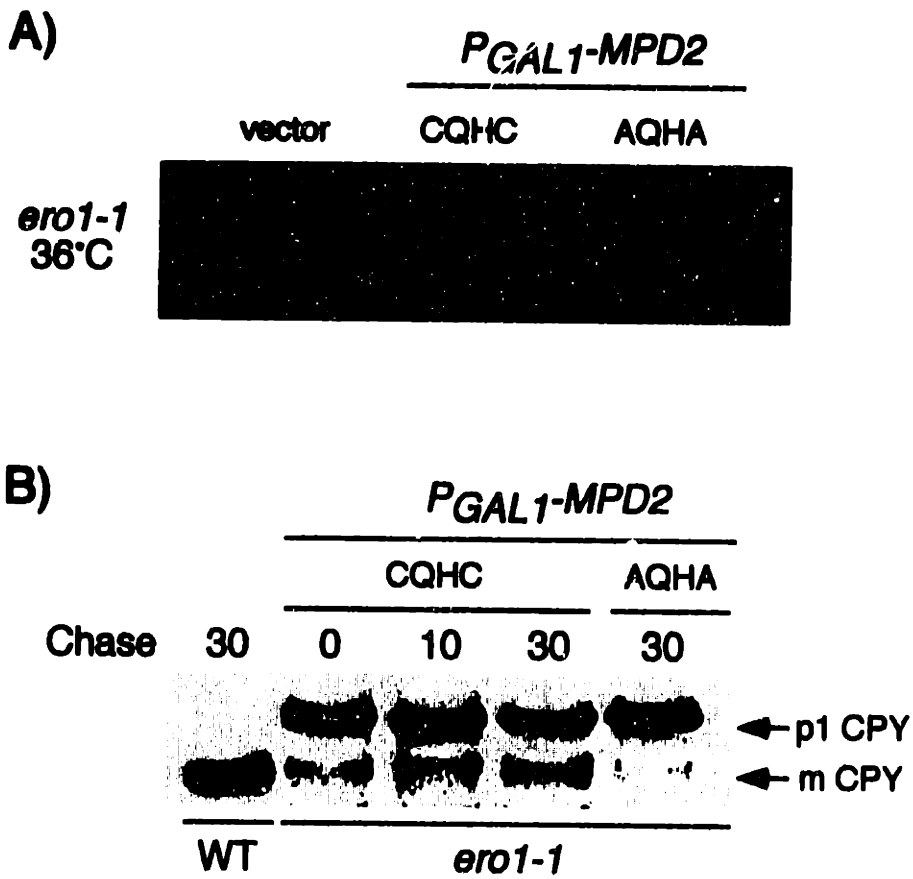

A)
P_{GAL1}-MPD2
vector
CQHC
AQHA
ero1-1
36°C
B)
P_{GAL1}-MPD2
CQHC
AQHA
Chase
30
0
10
30
30
p1 CPY
m CPY
WT
ero1-1

Figure 6

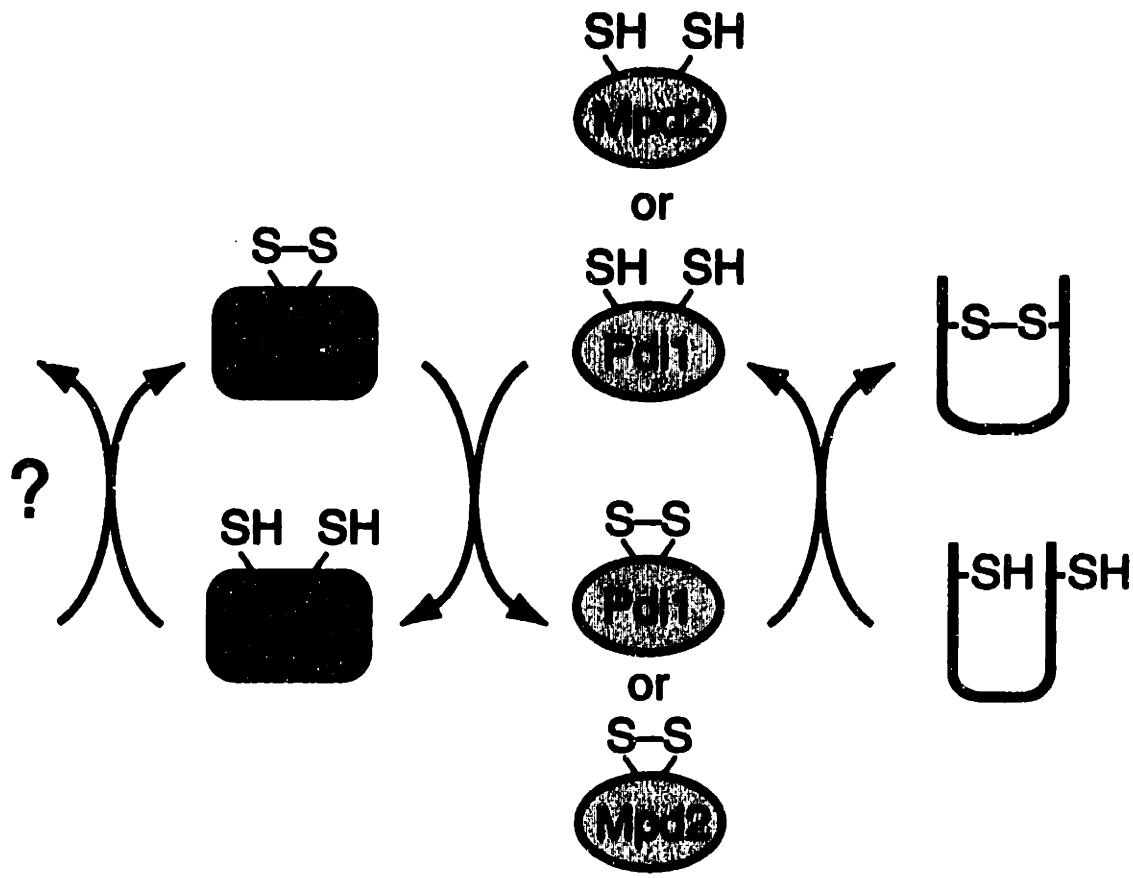


TABLE 1

Yeast Strains

Strain	Genotype	Source
CKY10	*MATa ura3-52 leu2-3,112*	Kaiser Lab Collection
CKY39	*MATα sec12-4 ura3-52 his4-619*	"
CKY263	*MATa ura3-52 leu2-3,112 GAL2*	"
CKY559	*MATα ero1-1 ura3-52 leu2-3,112*	"
CKY598	*MATa ero1-1 ura3-52 leu2-3,112 GAL2*	"
CKY612	*MATa mpd2-Δ::HIS3 ura3-52 leu2-3,112 his3-Δ200*	"
CKY564	*MATα pdi1-Δ::HIS3 ura3-1 leu2-3,112 his3-11,15 trp1-1 ade2-1 can1-100* [pCT37]	Tom Stevens

Chapter Four:

Two Pairs of Conserved Cysteines Are Required for Activity of Ero1p in Protein Disulfide Bond Formation

PREFACE

Pending the results of additional experiments, this chapter will be submitted for publication to the Journal of Cell Biology.

Abstract

The formation of protein disulfide bonds in the endoplasmic reticulum (ER) requires two essential proteins, Ero1p and protein disulfide isomerase (PDI). PDI, a luminal enzyme, serves as a direct oxidant for newly-synthesized secretory proteins, and is efficiently re-oxidized in vivo by Ero1p, an ER membrane protein. Here a mutational analysis of *ERO1* identifies two pairs of conserved, vicinal cysteines essential for the oxidative function of Ero1p. Cys100-Cys105 and Cys352-Cys355 of Ero1p are critical for cell viability and for oxidative folding of the vacuolar protease carboxypeptidase Y (CPY). The efficient capture of mixed-disulfides between Ero1p and PDI also requires these four cysteines. Cys100 of Ero1p appears most important for thiol-disulfide exchange with PDI, suggesting the preferential attack of this residue by the active-site cysteines of PDI. The Cys352-Cys355 disulfide of Ero1p may serve to re-oxidize the Cys100-Cys105 cysteine pair, possibly through an intramolecular thiol-disulfide exchange reaction. Maintenance of the oxidized redox state of Ero1p also requires Cys100-Cys105 and Cys352-Cys355. These results define the redox-active sites of Ero1p and illuminate the mechanism of Ero1p function.

Introduction

The formation of native intramolecular disulfide bonds is crucial for the folding and stability of many secreted proteins. In eukaryotic cells, this process is restricted to the lumen of the endoplasmic reticulum (ER) (Braakman et al., 1991), where relatively oxidizing redox conditions are sustained (Hwang et al., 1992). The oxidized redox state of the ER is reflected by a low intraluminal [GSH]/[GSSG] ratio of approximately 2:1. The [GSH]/[GSSG] ratio in the reducing environment of the cytosol is 10 to 100 fold higher (Hwang et al., 1992). Although GSSG is relatively abundant the ER, GSSG is not required as a source of oxidizing equivalents for protein disulfide bond formation in vivo (Frand and Kaiser, 1998, Cuozzo and Kaiser,

1999, Bader et al., 1999).

Protein disulfide bond formation is catalyzed both in vitro and in vivo by thiol-disulfide oxidoreductases of the thioredoxin superfamily, enzymes that possess redox-active cysteines in the characteristic motif CXXC (Holmgren, 1968, Martin, 1995). Protein disulfide isomerase (PDI) is a member of the thioredoxin superfamily abundant in the ER lumen (reviewed by Ferrari and Soling, 1999). In vitro, this enzyme catalyzes dithiol oxidation, disulfide reduction, or disulfide isomerization depending on the nature of the substrate protein and the redox conditions of the assay (reviewed by Freedman et al., 1994).

In yeast cells, PDI serves as the primary catalyst for disulfide bond formation and isomerization in secreted proteins. PDI engages directly in thiol-disulfide exchange with substrate proteins in the ER, as shown by the detection of mixed-disulfides between PDI and newly-synthesized carboxypeptidase Y (CPY) (Frand and Kaiser, in press). The *PDI1* gene of yeast is essential for cell viability and for oxidative folding of CPY (LaMantia and Lennarz, 1993). The ability of active-site mutants of PDI that retain isomerase activity but lack detectable oxidase activity in vitro to rescue the inviability of *pdi1Δ* cells has revealed the isomerase activity of PDI to be essential for cell growth (Laboissière et al., 1995). Nevertheless, the defect in CPY folding observed in PDI-deficient cells can be traced to a defect in the *formation* of disulfide bonds, demonstrating that PDI performs an extensive and critical role as an oxidase in vivo (Frand and Kaiser, in press). Consistent with this activity, PDI is predominantly oxidized in wild-type cells (Frand and Kaiser, in press). Moreover, active-site mutations that may perturb the redox potential of PDI are associated with hypersensitivity to the reductant DTT (Holst et al., 1997).

The re-oxidation of PDI depends upon Ero1p, a novel but conserved protein localized to the ER membrane (Frand and Kaiser, in press, Frand and Kaiser, 1998, Pollard et al., 1998). Direct thiol-disulfide exchange between these proteins is indicated by the capture of PDI-Ero1p mixed-disulfides in vivo (Frand and Kaiser, in press). Moreover, PDI is found entirely in the reduced state in the conditional *ero1-1* mutant (Frand and Kaiser, in press). Ero1p also oxidizes Mpd2p, a PDI-homolog

residing in the yeast ER (Frand and Kaiser, in press).

Several additional observations suggest that Ero1p serves as the predominant source of oxidizing equivalents utilized during protein disulfide bond formation in the ER. Although mutation of *ERO1* disrupts the formation of disulfide bonds in newly-synthesized secretory proteins, the addition of a thiol oxidant to the growth medium can restore growth and secretion to *ero1* mutants (Frand and Kaiser, 1998). Moreover, overexpression of *ERO1* confers resistance to DTT, indicating that Ero1p levels determine the oxidizing capacity of the cell (Frand and Kaiser, 1998, Pollard et al., 1998). Ero1p activity is coupled to the production of oxidized glutathione in vivo, possibly as a consequence of driving protein disulfide bond formation (Cuozzo and Kaiser, 1999). Together, these findings indicate that the flux of oxidizing equivalents into the ER lumen depends upon oxidation of Ero1p.

The recent discovery that Ero1p engages directly in thiol-disulfide exchange with PDI implied the existence of redox-active disulfide bonds in Ero1p. However, the lack of sequence homology between Ero1p and known oxidoreductases impeded the identification of these disulfides. We therefore undertook a mutational analysis of *ERO1* in order to assess the functional importance of the seven conserved cysteines in Ero1p. Our results define the redox-active sites of Ero1p by locating two pairs of conserved, vicinal cysteines essential for the activity of Ero1p in protein disulfide bond formation.

Results

Two Pairs of Conserved, Vicinal Cysteines Are Essential for the Oxidative Function of Ero1p In Vivo.

Of the fourteen cysteines present in yeast Ero1p, seven are absolutely conserved among the eukaryotic Ero1 sequence homologs identified to date, and these residues define positions 90, 100, 105, 208, 349, 352, and 355 of yeast Ero1p (Figure 1A). The three N-terminal cysteines are found in a moderately conserved

region of Ero1p, while the remaining four cysteines occupy two highly conserved regions, in which the human and yeast Ero1 homologs are up to 65% identical (Figure 1B, Frand and Kaiser, 1998, Pollard et al., 1998). Seven new alleles of *ERO1* were generated, each specifying alanine in place of one conserved cysteine. To facilitate detection of the mutant proteins, the desired mutations were introduced in the context of *ERO1-myc*, a functional, epitope-tagged version of *ERO1* (Frand and Kaiser, 1998). We refer to these mutant proteins as CAX Ero1p-myc, and to the corresponding alleles as *ero1-AX-myc*, where X designates the position of the alanine substitution in the primary sequence of Ero1p.

To assess the functional importance of each conserved cysteine in Ero1p, each alanine substitution mutant of *ERO1* was tested for complementation of the conditional *ero1-1* mutant. Expression of *ero1-A100-myc*, *ero1-A105-myc*, *ero1-A352-myc*, or *ero1-A355-myc* failed to restore growth to *ero1-1* cells at restrictive temperature (38°C). In contrast, expression of *ero1-A90-myc*, *ero1-A208-myc*, or *ero1-A349-myc* rescued the growth defect of these cells (Figure 2A). In yeast, the accumulation of misfolded proteins in the ER promotes the induction of ER chaperones and oxidoreductases in a manner dependent upon the ER transmembrane kinase Ire1p (Cox et al., 1993). This pathway, termed the Unfolded Protein Response (UPR), becomes activated in cells compromised for Ero1p activity, and thereby triggers overexpression of *ERO1*. The complementing activities of *ero1-A90-myc*, *ero1-A100-myc*, *ero1-A208-myc*, and *ero1-A349-myc* were therefore assessed in an *ire1Δ* background to prevent induction of these alleles by the UPR. Inactivation of the UPR was necessary to reveal the phenotype associated with *ero1-A100-myc*, since this mutant partially rescued the temperature-sensitive growth of *ero1-1* cells in an *IRE1*+ strain. The complementing activity of wild-type *ERO1-myc* was also verified in the *ero1-1 ire1Δ* double mutant (Figure 2A).

The alanine substitution mutants of *ERO1* were further tested for their ability to rescue the inviability associated with a chromosomal deletion of *ERO1*. A diploid heterozygous for a *LEU2*-marked disruption allele of *ERO1* (*ero1-Δ1-500::LEU2*) was constructed lacking other functional alleles of *LEU2* or *URA3*. The heteroallelic

diploid was transformed with *URA3*-marked plasmids carrying each *ero1* allele, and sporulated. The recovery of Leu$^+$ Ura$^+$ spore clones indicated that the episomal *ero1* allele could restore viability to *ero1-Δ1-500::LEU2* spores. In a pattern identical to that observed for complementation of the conditional *ero1-1* mutant, *ero1-A105-myc*, *ero1-A352-myc*, and *ero1-A355-myc* were not observed to rescue the inviability of *ero1Δ-1-500* spores, whereas *ero1-A90-myc*, *ero1-A208-myc*, *ero1-A349-myc*, and *ERO1-myc* did restore growth to these cells. The *ero1-A100-myc* allele failed to restore viability to *ero1-Δ1-500* spores in an *ire1Δ* background, but could partially restore growth to such cells in an *IRE1+* strain (data not shown).

The oxidizing capacity of *IRE1$^+$* cells expressing only *ero1-A100-myc* was examined more closely by assessing the sensitivity of these cells to the reductant DTT. Lawns of CKY602 (*ero1-Δ1-500::LEU2 IRE1* pAF131) were plated on rich medium and 30 μM of DTT were applied to each lawn on a sterile filter disk. CKY600 was unable to grow inside a zone 38 cm in diameter surrounding the DTT source (Figure 2B). A similar zone of growth inhibition was observed for the conditional *ero1-1* mutant at semi-permissive temperature (30°C). In contrast, *ero1-Δ1-500::LEU2* cells expressing wild-type *ERO1-myc* (CKY600) were unable to grow inside a zone only 25 cm in diameter surrounding the DTT source (Figure 2B). These results show that *ero1-A100-myc* can not restore the oxidizing capacity of *ero1-Δ1-500::LEU2* cells, indicating that Cys100 of Ero1p is essential for the oxidative function of *ERO1*.

To allow for the detection of subtle decreases in cellular oxidizing capacity associated with mutation of Cys90, Cys208, or Cys349 of Ero1p, we further examined the DTT sensitivity of *ero1-Δ1-500::LEU2* cells expressing *ero1-A90-myc*, *ero1-A208-myc*, or *ero1-A349-myc*. These strains displayed approximately the same zone of growth inhibition as CKY600 (data not shown), indicating that each of these *ero1* mutants could provide the oxidizing activity of *ERO1*.

The replacement of a single cysteine in Ero1p that is normally disulfide-bonded could theoretically convert the second cysteine of the pair into an abnormally

reactive species. This possibility raised the concern that mutations in *ERO1* associated with loss-of-function phenotypes could actually specify proteins that disrupt protein oxidation in the ER in a dominant fashion. We therefore examined the DTT sensitivity of wild-type strains (CKY263) overexpressing *ero1-A100-myc*, *ero1-A105-myc*, *ero1-A352-myc*, or *ero1-A355-myc*. In a halo assay performed under selective growth conditions, these strains displayed an equivalent sensitivity to DTT as untransformed wild-type cells (data not shown). This observation disfavors the possibility that these *ero1* mutants interfere with protein disulfide bond formation in the ER.

Together, these results show that of the seven absolutely conserved cysteines in Ero1p, only Cys100-105 and Cys352-355 are essential for the oxidative function of Ero1p. Moreover, Cys105, Cys352, and Cys355 of Ero1p are required for cell viability. Although Cys100 of Ero1p is also important for cell viability, induction of the UPR may circumvent the requirement for this residue in a manner dependent upon overproduction of either CA100 Ero1p itself or other ER oxidoreductases. The identification of two pairs of conserved, vicinal cysteines required for the activity of Ero1p implicates these cysteines in the formation of two redox-active sites in Ero1p .

Efficient Thiol-disulfide Exchange with PDI and Mpd2p Requires Cys100-Cys105 and Cys352-Cys355 of Ero1p.

The capture of mixed-disulfides between Ero1p-myc and PDI has recently provided evidence that Ero1p engages directly in thiol-disulfide exchange with PDI in vivo (Frand and Kaiser, in press). These complexes are detected when cells overproducing Ero1p-myc as well as PDI are treated directly with trichloroacetic acid (TCA). This reagent rapidly lowers intracellular pH, thereby inhibiting further thiol exchange in vivo and precipitating cellular proteins. Overproduction of a CGHS-CGHS active-site mutant of PDI enhances detection of these complexes, presumably because this form of PDI is defective in the resolution of mixed-

disulfides in vivo (Frand and Kaiser, in press).

To evaluate the role of the conserved cysteines of Ero1p in thiol-disulfide exchange with PDI, the efficiency of mixed-disulfide capture between CGHS-CGHS PDI and Ero1p-myc was assessed in strains overproducing each alanine substitution mutant of Ero1p. This assay was performed first in an *ero1-1* strain background (CKY598) so that the properties of each Ero1p variant could be assessed in isolation. Cells were radiolabeled with [^{35}S]-methionine at the restrictive-temperature for *ero1-1* (38°C) and then treated directly with TCA. Free thiols were then covalently modified with N-ethylmaleimide (NEM) prior to immunoprecipitation with anti-myc antibody under non-reducing but denaturing conditions. The anti-myc immunoprecipitates, which would contain PDI-Ero1p mixed-disulfides, were then reduced by DTT and divided prior to re-immunoprecipitation with either anti-PDI or anti-myc antibody. The efficiency of mixed-disulfide capture was expressed as the ratio of PDI trapped to total Ero1p-myc, standardized to the value obtained from cells overproducing wild-type Ero1p-myc.

Substitution of Cys100, Cys105, Cys352, or Cys355 of Ero1p-myc with alanine decreased the efficiency of trapping PDI in mixed-disulfides 75% to 96% (Figure 3A). This result further implicates Cys100-Cys105 and Cys352-Cys355 of Ero1p in the formation of redox-active disulfide bonds, one or both of which could engage directly in thiol-disulfide exchange with PDI. However, a diminished efficiency of mixed-disulfide capture could also result from an overall decrease in the redox state of the ER lumen associated with the complete loss of *ERO1* function in these cells. To help distinguish the functions of Cys100- Cys105 and Cys352-Cys355, the capture of mixed-disulfides was therefore assayed in a wild-type strain background, where the chromosomal allele of *ERO1* would sustain the oxidized redox state of the ER lumen. In this context, mixed-disulfides containing CA100 Ero1p-myc were captured only 30% as efficiently as those containing wild-type Ero1p-myc. In contrast, PDI was captured 54% to 76% as efficiently with CA105, CA352, or CA355 Ero1p-myc as with wild-type Ero1p-myc. These results suggest that the active-site cysteines of PDI may preferentially attack Cys100 of Ero1p, while

Cys105, Cys352, and Cys355 of Ero1p play auxiliary roles in thiol-disulfide exchange with PDI. Following disulfide interchange with PDI, the Cys352-Cys355 disulfide of Ero1p may, directly or indirectly, serve to re-oxidize the Cys100-Cys105 cysteine pair. This analysis was extended by examining the interaction of each Ero1p mutant with an additional substrate, the PDI-homolog Mpd2p. Mpd2p-Ero1p mixed-disulfides (125 kDa) are readily detected following TCA treatment of wild-type cells overproducing a CQHA active-site mutant of Mpd2p along with Ero1p-myc (Figure 3C, Frand and Kaiser, in press). Substitution of Cys100 of Ero1p with alanine abolished the capture of mixed-disulfides with CQHA Mpd2p. Mixed-disulfides containing CA105, CA352, or CA355 Ero1p-myc were captured only 13% to 35% as efficiently as those containing wild-type Ero1p-myc (Figure 3C). These results corroborate our findings with PDI and affirm the importance of the Cys100-Cys105 and Cys352-Cys355 cysteine pairs of Ero1p for thiol-disulfide exchange with thioredoxin-like oxidoreductases. An explanation for the higher sensitivity of trapping mixed-disulfides with Mpd2p may require additional information concerning the biochemical properties of this enzyme.

In experiments with CGHS-CGHS PDI and CQHA Mpd2p, mixed-disulfides containing CA90 or CA349 Ero1p-myc were captured 1.4 to 1.8-fold more efficiently than mixed-disulfides containing wild-type Ero1p-myc (Figure 3). These mixed-disulfides also migrated somewhat anomalously during non-reducing SDS-PAGE (Figure 3C and data not shown). These observations raise the intriguing possibility that Cys90 and Cys349 of Ero1p may normally negatively regulate mixed-disulfide capture, perhaps by expediting the release of mixed-disulfides from Ero1p or inhibiting their formation.

Together, these results show that efficient thiol-disulfide exchange between Ero1p and PDI or Mpd2p requires Cys100-Cys105 and Cys352-Cys355 of Ero1p. Of these four cysteines, Cys100 of Ero1p is most important for the capture of mixed-disulfides in living cells.

Oxidative Protein Folding in the ER Requires Cys100-Cys105 and Cys352-Cys355 of Ero1p.

To assay the importance of Cys100-Cys105 and Cys352-Cys355 of Ero1p for oxidative protein folding in the ER, the processing of newly-synthesized carboxypeptidase (CPY) was monitored in *ero1-1* cells expressing each mutant. CPY normally undergoes oxidative protein folding in the ER to achieve a native structure with five intramolecular disulfide bonds (Endrizzi et al., 1994, Jämsä et al., 1994). However, in the *ero1-1* conditional mutant, the formation of these disulfide bonds is disrupted and consequently CPY is retained in the ER in the p1 precursor form (Frand and Kaiser, 1998). When *ero1-1* cells expressing *ero1-A100-myc*, *ero1-A105-myc*, *ero1-A352-myc*, or *ero1-A355-myc* were pulse-labeled at restrictive temperature, newly-synthesized CPY was fully retained in the ER in p1 form (Figure 4). In contrast, CPY was processed to the mature, vacuolar form in *ero1-1* cells rescued by *ERO1-myc* (Figure 4). These results show that Cys100-Cys105 and Cys352-Cys355 of Ero1p are required for efficient disulfide bond formation in CPY. The observation that *ero1* mutants defective in thiol-disulfide exchange with PDI are also defective in the oxidative folding of CPY affirms that the transfer of oxidizing equivalents from Ero1p to PDI is an important step in protein disulfide bond formation in vivo.

Cys100-Cys105 and Cys352-Cys355 Are Required to Sustain the Oxidized Redox State of Ero1p in Vivo.

Since our data suggested that Cys100-Cys105 and Cys352-Cys355 of Ero1p could participate in the formation of redox-active disulfide bonds, the oxidation state of CA100, CA105, CA352 and CA355 Ero1p-myc was examined upon overproduction of each mutant in wild-type cells. Following TCA-precipitation, the oxidation state of each protein was assessed by the modification of free thiols with AMS. The apparent molecular mass of a large portion of each Ero1p mutant increased by 15

kDa upon modification with AMS, indicating that each protein partitioned into the reduced form in vivo (Figure 5). Wild-type Ero1p-myc reduced in vivo by the treatment of cells with 10 mM DTT reacted with AMS to a slightly greater extent, consistent with AMS modification of one additional cysteine residue (Figure 5, Frand and Kaiser, in press). In contrast, wild-type Ero1p-myc, even when overproduced, could not be modified by AMS and therefore appeared fully oxidized in vivo (Figure 5, Frand and Kaiser, in press). Although CA355 Ero1p-myc was not detected in the oxidized form in this assay, the ability of CA355 Ero1p-myc to form mixed-disulfides with CGHS-CGHS PDI suggested that an oxidized form of this protein did exist in vivo. The oxidized form of CA355 Ero1p-myc may therefore be extremely short-lived, or may be represented entirely by mixed-disulfides of higher molecular weight. Together, these results show that Cys100-Cys105 and Cys352-Cys355 of Ero1p are important for maintenance of the oxidized redox state of Ero1p in vivo, consistent with the model that these residues form redox-active disulfides in Ero1p. Oxidation of either cysteine pair may be sufficient to maintain a portion of Ero1p in the oxidized form detected by this assay.

Discussion

The formation of protein disulfide bonds in the ER requires two essential proteins, Ero1p and PDI. Recent work has revealed a pathway for disulfide bond formation wherein oxidizing equivalents are transferred from Ero1p to PDI and then to substrate proteins through direct thiol-disulfide exchange reactions. Although the elucidation of this pathway implied the existence of at least one redox-active disulfide bond in Ero1p, this disulfide had not been identified. In this work, we show that two pairs of conserved, vicinal cysteines, located at positions 100-105 and 352-355 of yeast Ero1p, are essential for the oxidative function of Ero1p. These cysteines are important for cell viability, for oxidative folding of CPY, and for efficient thiol-disulfide exchange between Ero1p and PDI. This correlation affirms the importance of disulfide transfer from Ero1p to PDI for protein oxidation in vivo and

defines Cys100-Cys105 and Cys352-Cys355 as the redox-active sites in Ero1p.

The spacing between Cys100 and Cys105, and between Cys352 and Cys355, in the primary sequence of Ero1p immediately suggested assignment of these residues as redox-active disulfides. Cys100 and Cys105 of yeast Ero1p occur within the sequence GL**C**LNRA**C**SV (Figure 1). A precedent for a similar configuration of a redox-active disulfide is provided by glutathione reductase, an enzyme catalyzing the NADPH-dependent reduction of oxidized glutathione through an FAD cofactor and an enzymic disulfide found in the motif GT**C**VNVG**C**VP (see Kuriyan et al., 1991). The N-terminal cysteine of this motif interacts directly with oxidized glutathione. The Cys352-Cys355 cysteine pair of yeast Ero1p may define a canonical CXXC motif, the redox-active site found in thiol-disulfide oxidoreductases of the thioredoxin superfamily (see Martin et al., 1995, Chivers et al., 1997). Although Ero1p does not display obvious sequence homology to thioredoxin, the protein may nevertheless contain a thioredoxin-like fold, since primary sequence data alone may be insufficient to identify this domain (Ellis et al., 1992). However, a CXXC motif could also function in Ero1p in the absence of a thioredoxin-like fold, as observed for DsbB (Jander et al., 1994). The consensus sequence corresponding to residues 352-255 of yeast Ero1p is C(D/E)KC. The influence of the internal (D/E)K residues on the redox properties of Ero1p remains to be evaluated.

Cys352 and Cys355 of yeast Ero1p appear in the context of a highly conserved region containing a third vicinal cysteine at position 349 (Figure 1B). As the motif CXXCXXC can specify part of an iron-sulfur cluster in iron-binding proteins (Beinert, 1990), the existence of this third cysteine had led to the suggestion that Ero1p would function as a redox-sensor through the conjugation of iron (Pollard et al., 1998). The observation that Ero1p function does not require Cys349 disfavors, but does not exclude, this possibility.

The pathway recently defined for protein oxidation in the ER appears analogous in many respects to the pathway for disulfide bond formation in the E. coli periplasm. In E. coli, the thioredoxin-like oxidoreductase DsbA serves as the principal catalyst of disulfide bond formation in periplasmic proteins (Bardwell et. al,

1991). DsbA is re-oxidized directly by the cytoplasmic membrane protein DsbB (Bardwell et. al, 1993, Dailey and Berg, 1993, Missiakas et al., 1993). Mammalian PDI complements the E. coli dsbA mutant (Humphreys et al, 1995). Further, the properties of S. cerevisiae *ERO1* resemble those of E. coli dsbB. Mutations in either *ERO1* or dsbB can be suppressed by an exogenous oxidant, while overexpression of either gene confers resistance to DTT (Bardwell et. al., 1993, Dailey and Berg, 1993, Missiakas et al., 1993, Frand and Kaiser, 1998, Pollard et al., 1998). Most importantly, Ero1p and E. coli DsbB are both membrane proteins implicated in the direct oxidation of soluble, thioredoxin-like oxidoreductases (Bardwell et al., 1993, Frand and Kaiser, in press).

The identification of two pairs of vicinal cysteines required for the oxidative function of Ero1p invites further comparison of Ero1p to DsbB. Although DsbB is not likely to contain a thioredoxin-like fold, the enzyme nevertheless possesses two redox-active disulfide bonds essential for its oxidizing activity towards DsbA in vivo and in vitro (Jander et al., 1994, Bader et al., 1999). The cysteine pairs forming these bonds, Cys41-Cys44 and Cys104-Cys130, are located in distinct periplasmic loops of DsbB (Jander et al., 1994). The Cys104-Cys130 disulfide may directly engage in thiol-disulfide exchange with DsbA, since the formation of mixed disulfides between DsbA and DsbB specifically requires Cys104 of DsbB (Guilhot et al., 1995, Kishigami et al., 1995, Kishigami and Ito, 1996). The N-terminal Cys41-Cys44 disulfide defines a redox-active CVLC motif, and is thought to re-oxidize the Cys104-Cys130 cysteine pair, possibly through an intramolecular thiol-disulfide exchange reaction (Kishigami and Ito, 1996, Guilhot et al., 1995). In accordance with this view, substitution mutants of DsbB lacking Cys41, Cys44, or Cys130 also display defects in mixed-disulfide formation with DsbA33S, but only when cells are grown in the absence of small molecule oxidants, such as GSSG (Kishigami and Ito, 1996, Guilhot et al., 1995). Moreover, loss of the Cys41-Cys44 disulfide bond results in reduction of Cys104 and Cys130 in vivo (Kobayashi and Ito, 1999). The Cys41-Cys44 cysteine pair of DsbB is maintained in the oxidized state in vivo in a manner dependent upon respiratory chain function and oxygen (Kobayashi and Ito, 1999,

Bader et al., 1998).

A similar mechanism can be envisioned for the function of Ero1p. Ero1p mutants in which Cys100, Cys105, Cys352, or Cys355 are replaced with alanine are defective in the formation of CGHS-CGHS PDI-Ero1p mixed disulfides in *ero1-1* cells. Cys100 of Ero1p is most important for thiol-disulfide exchange with PDI, since only CA100 Ero1p is severely defective in mixed-disulfide formation when expressed in wild-type cells. Further, substitution of Cys100 completely blocks mixed-disulfide formation with Mpd2p. These results suggest that the Cys100-Cys105 disulfide of Ero1p is preferentially transferred to PDI-homologs. The Cys352-Cys355 disulfide may then serve to re-oxidize the Cys100-Cys105 cysteine pair. The latter step could proceed through an intramolecular thiol-disulfide exchange reaction, or possibly through an intermolecular exchange reaction involving a second molecule of Ero1p.

According to this model, the mixed-disulfides detected between PDI and either CA352 or CA355 Ero1p in wild-type cells could form following oxidation of the Cys100-Cys105 disulfide by wild-type Ero1p or an alternative disulfide donor. In lieu of the Cys100-Cys105 disulfide, PDI may attack other intra- or intermolecular disulfides present in the cysteine to alanine substitution mutants of Ero1p. The formal possibility that mutation of Cys100 serves to expedite the release of PDI-Ero1p mixed disulfides, rather than to inhibit their formation, can not be excluded from the present data.

Ero1p itself may become oxidized at the Cys352-Cys355 cysteine pair. Oxidation of these cysteines will introduce oxidizing equivalents into the lumen of the ER, and may thereby complete the essential function of Ero1p. In the absence of Cys100 of Ero1p, these oxidizing equivalents could ultimately be transferred to substrate proteins through alternative, less efficient pathways involving PDI or glutathione. Induction of the UPR may circumvent the requirement for Cys100 for cell viability by increasing the efficiency of these alternative pathways through the overexpression of Ero1p or PDI.

The mechanism responsible for re-oxidizing Ero1p remains a mystery. However, the identification of the redox-active sites of Ero1p should expedite

investigation of the mechanism responsible for re-oxidation of Ero1p, and allow the establishment of biochemical assays for Ero1p function.

Methods

S. cerevisiae strains were grown and genetically manipulated using standard techniques (Kaiser et al., 1994). YPD is rich medium with 2% glucose, and YEP Raf/Gal is rich medium with 2% raffinose and 2% galactose. SC is minimal media with 2% glucose and SC Raf/Gal is rich medium with 2% raffinose and 2% galactose. 1 OD_{600} U corresponds to $2X10^7$ cells. Table 1 lists the genotypes of strains used in this study.

Plasmids and strain constructions

The *ero1-A90-myc*, *ero1-A100-myc*, *ero1-A105-myc*, *ero1-A208-myc*, *ero1-A349-myc*, *ero1-A352-myc*, and *erc1-A355-myc* alleles were each generated by site-directed mutagenesis on pAF82 (*CEN URA3 ERO1-myc*, Frand and Kaiser, 1998), creating pAF98, pAF131, pAF122, pAF99, pAF120, pAF96, and pAF95, respectively. The genomic inserts from these plasmids were cloned into pRS305-2μ (2μ *LEU2*, Sikorski and Hieter, 1989) by homologous recombination *in vivo*, generating pAF124, pAF125, pAF126, pAF127, pAF128, pAF129, and pAF130, respectively. The *ero1-A90-myc*, *ero1-A100-myc*, *ero1-A208-myc*, and *ero1-A349-myc* alleles were further cloned into pRS315 (*CEN LEU2*) by homologous recombination in CKY605 to generate pAF153, pAF154, pAF155, and pAF156. Plasmids pAF89 (2μ *LEU2 ERO1-myc*) and pAF85 (*CEN LEU2 ERO1-myc*) are described in Frand and Kaiser, 1998.

To generate a complete chromosomal deletion of *ERO1*, a 2.3 kb BamHI-HindIII fragment containing 1.5 kb of *ERO1* coding sequence as well as 0.8 kb of 5' sequence devoid of other open reading frames was released from pAF82 and replaced with a 2.0 kb BamHI-HindIII fragment from pJJ252 containing the *LEU2* marker (Jones and Prakash, 1990). The *ero1-Δ1-500::LEU2* allele was then

isolated as an SphI-KpnI fragment and introduced into a *ura3-52, leu2-3,112* diploid. Sporulation of a Leu^+ transformant, CKY599, produced no more than 2 viable, Leu^- spores per tetrad. Transformation of CKY599 with pAF82 (*CEN URA3 ERO1-myc*) prior to sporulation allowed the recovery of viable, Leu^+, Ura^+ spore clones dependent on the episomal *ERO1* allele for viability and therefore unable to grow on media containing 5-fluoroorotic acid (5-FOA, Toronto Research Laboratories). CKY600 (*ero1-Δ1-500::LEU2* pAF82) represents one such clone. CKY601, CKY602, CKY603, and CKY604 were generated in a similar fashion by sporulation of CKY599 transformants carrying pAF98, pAF131, pAF99, or pAF120, respectively.

Complementation of *ero1* loss-of-function phenotypes

To test for complementation of the temperature-sensitive growth defect of *ero1-1* cells, CKY558 (*ero1-1*) was transformed with pAF122, pAF96, or pAF95, and CKY605 (*ero1-1 ire1Δ ::URA3*) was transformed with pAF85, pAF153, pAF154, pAF155, or pAF156. The strains were grown selectively to exponential phase, plated on YPD at a density of 1 OD_{600} U/ml, and incubated at 38°C overnight. Prior to testing, CKY605 transformants were cultured exclusively at 24°C, since this strain is inviable at temperatures above 29°C. To test for rescue of the inviability associated with a chromosomal deletion of *ERO1*, CKY599 *(ero1-Δ1-500::LEU2/ERO1 leu2 -3,112/leu2 -3,112 ura3-52/ura3-52*) was transformed with pAF82, pAF98, pAF131, pAF122, pAF99, pAF120, pAF96, or pAF95 and then sporulated. The recovery of Leu^+ Ura^+ spore clones that were unable to grow on plates containing 5-FOA indicated that the episomal *ERO1* allele could restore growth to *ero1-Δ1-500::LEU2* spores. An episomal *ero1* allele was considered to lack rescuing activity when dissection of at least 18 asci produced tetrads with no more than 2 viable, Leu^- spores, even though Ura^+ segregants could be identified in several tetrads, verifying inheritance of the covering plasmid. To test the ability of *ero1-A100-myc* to rescue the inviability of *ero1-Δ1-500::LEU2 ire1Δ ::URA3* spores,

CKY602 (*ero1-Δ1-500::LEU2* pAF131) was crossed to CKY561 (*ire1Δ::URA3*) and the resulting diploid sporulated. Tetratype and nonparental ditype tetrads inheriting pAF131 were identified by the segregation of Leu^- Ura^+ Ts^+ clones viable on media containing 5-FOA. In these tetrads, phenotypes of the sister spores consistently allowed assignment of the *ero1-Δ1-500::LEU2 ire1Δ ::URA3* genotype to inviable spores.

Kinetic analyses of CPY transport and halo assays for DTT sensitivity were performed as described (Frand and Kaiser, 1998).

Trapping Mixed-Disulfides between Ero1p-myc and PDI or Mpd2p

The capture of mixed-disulfides between Ero1p-myc and either CGHS-CGHS PDI or CQHA Mpd2p was performed essentially as described (Frand and Kaiser, in press) except that primary anti-myc immunoprecipitates were prepared corresponding to only 10 OD_{600} U of cells. 10% of each sample was saved for analysis by non-reducing SDS-PAGE while the remainder was reduced by boiling in 100 mM DTT. 15% of each sample was then re-immunoprecipitated with anti-myc antibody and 85% re-immunoprecipitated with anti-PDI antibody. Strains derived from CKY598 (*ero1-1*) were grown to exponential phase at 24°C and then shifted to 38°C for 12 min prior to radiolabeling. After harvesting by centrifugation, these cells were suspended in 100 µl of growth medium and returned to 38°C for 5 min before the addition of 10 µl of 100% (w/v) trichloroacetic acid (TCA). The oxidation state of Ero1p-myc was assessed as previously described (Frand and Kaiser, in press).

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Figure Legends

Figure 1 Conserved Cysteines in Ero1p

A) Seven cysteines are absolutely conserved among the eukaryotic sequence homologs of Ero1. These define positions 90, 100, 105, 208, 349, 352, and 355 of yeast Ero1p.

B) Sequence of Ero1 homologs surrounding Cys100-Cys105 and Cys352-Cys355 of yeast Ero1p.

Figure 2 Cys100-Cys105 and Cys352-Cys355 of Ero1p Are Essential for the Oxidative Function of *ERO1* in vivo

A) Complementation of the conditional *ero1-1* mutant by alleles of *ERO1* specifying alanine substitution mutants of Ero1p. Strains were incubated at restrictive temperature (38°C) overnight on YPD. Alleles of *ERO1* with detectable rescuing activity were expressed in *ireΔ* cells in order to prevent induction of *ERO1* by the Unfolded Protein Response. Transformants of CKY605 (*ero1-1 ireΔ::URA3*) contained: WT, pAF85 (*CEN ERO1-myc*); 90, pAF153 (*CEN ero1-A90-myc*); 100, pAF154 (*CEN ero1-A100-myc*); 208, pAF155 (*CEN ero1-A208-myc*); 349, pAF156 (*CEN ero1-A349-myc*). Transformants of CKY559 (*ero1-1*) contained: 105, pAF122 (*CEN ERO1-A105-myc*); 352, pAF96 (*CEN ERO1-A352-myc*); 355, pAF95 (*CEN ERO1-A355-myc*); vector, pRS316 (*CEN URA3).*

B) DTT sensitivity of the *ero1-Δ1-500::LEU2* strain expressing CA100 Ero1p-myc. Three lawns of CKY602 were plated on YPD and 10 µl of 3M DTT applied to each lawn in a filter disk. The average diameter of the zone of growth inhibition (mm) was measured after incubation at 30°C for 1.5 days. Lawns of CKY600 (*ero1-Δ1-500::LEU2* p*ERO1-myc*) and CKY559 (*ero1-1*) are shown for comparison.

Figure 3 Efficient Thiol-Disulfide Exchange with PDI and Mpd2p Requires Cys100-Cys105 and Cys352-Cys355 of Ero1p.

Cells overexpressing an alanine substitution mutant of *ERO1*-myc in addition to P_{GAL1}-*PDI1-CGHS-CGHS* (pAF132) were labeled with [^{35}S]-methionine and cysteine and then suspended in 10% TCA to block further thiol exchange in vivo. To isolate PDI-Ero1p mixed-disulfides, free thiols were modified with NEM and the sample immunoprecipitated with anti-myc antibody under non-reducing conditions. The immunoprecipitates were then reduced with 100 mM DTT and divided before re-immunoprecipitation with either anti-myc (1x loading) or anti-PDI (7.5x loading) antibody. Samples were resolved by SDS-PAGE and analyzed with a 445si phosphorimager. The efficiency of mixed-disulfide capture is expressed as the ratio of the intensity of PDI trapped to total Ero1p-myc per OD_{600} U of extract, and is standardized to the value obtained with wild-type Ero1p-myc. A) Strains derived from CKY598 (*ero1-1 GAL2*) were labeled at 38°C and maintained the following plasmids: WT, pAF89 (*2μ ERO1-myc*); 90, pAF124 (*2μ ero1-A90-myc*); 100, pAF125 (*2μ ero1-A100-myc*); 105, pAF126 (*2μ ero1-A105-myc*); 208, pAF127 (*2μ ero1-A208-myc*); 349, pAF128 (*2μ ero1-A349-myc*); 352, pAF129 (*2μ ero1-A352-myc*); 355, pAF130 (*2μ ero1-A355-myc*). B) Otherwise isogenic strains derived from CKY263 (wild-type *GAL2*) were labeled at 30°C. C) Mixed-disulfides between CQHA Mpd2p and alanine substitution mutants of Ero1p-myc were isolated from similar CKY263 transformants hosting pAF123 (P_{GAL1}-*MPD2-CQHA*) in place of pAF132. These complexes were resolved directly by non-reducing SDS-PAGE, and the efficiency of mixed-disulfide capture expressed as the ratio of the intensity of Mpd2p-Ero1p-myc complexes to free Ero1p-myc.

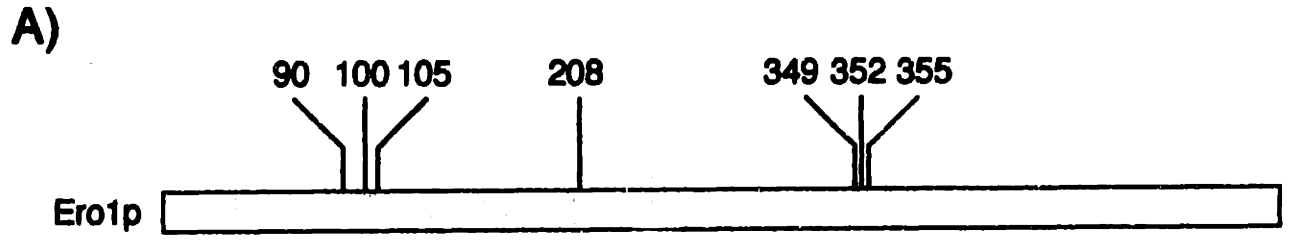
Figure 4 Oxidative Protein Folding of CPY Requires Cys100-Cys105 and Cys352-Cys355 of Ero1p.

Alleles of *ERO1* specifying alanine substitution mutants of Ero1p were expressed in the conditional *ero1-1* mutant and the processing of newly-synthesized CPY monitored after a 20 min pulse-labeling at 38°C. CPY was immunoprecipitated and the samples resolved by SDS-PAGE. The p1 (ER) and mature (vacuolar) forms of CPY are indicated. *ERO1-myc* and *ero1-A100-myc* were expressed in CKY605 (*ero1-1 ire1Δ*) to prevent induction of *ERO1* by the UPR; other alleles were expressed in CKY559 (*ero1-1*). The strains shown hosted: WT, pAF85 (*CEN ERO1-myc*); 100, pAF154 (*CEN ero1-A100-myc*); 105, pAF122 (*CEN ero1-A105-myc*); 352, pAF96 (*CEN ero1-A352-myc*); 355, pAF95 (*CEN ero1-A355-myc*); and -, no plasmid.

Figure 5 Cys100-Cys105 and Cys352-Cys355 Are Required to Maintain the Oxidized Redox State of Ero1p in vivo.

The oxidation state of CA100, CA105, CA352, and CA355 Ero1p-myc overproduced in wild-type cells (CKY263) was assessed by the precipitation of cellular proteins with TCA followed by the modification of free thiols with AMS. Samples were resolved by non-reducing SDS-PAGE and Ero1p-myc detected by immunoblotting. To provide a standard for the mobility of reduced Ero1p-myc, cells expressing *ERO1-myc* were treated with 5 mM DTT. The AMS-modified reduced and oxidized forms of Ero1p-myc are indicated. The strains shown hosted the following plasmids: WT, pAF89 (*2μ ERO1-myc*); 100, pAF125 (*2μ ero1-A100-myc*); 105, pAF126 (*2μ ero1-A105-myc*); 352, pAF129 (*2μ ero1-A352-myc*); 355, pAF130 (*2μ ero1-A355-myc*).

Figure 1



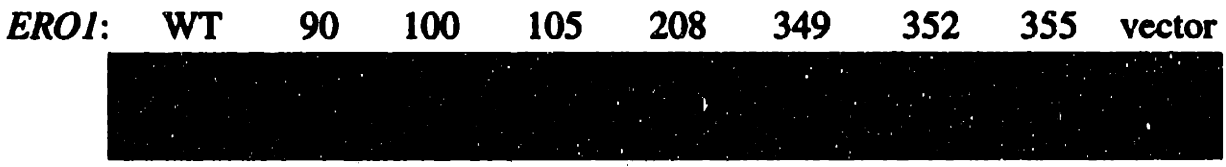
B)

S. cerevisiae (90)	S	F	D	A	N	D	G	L	L	N	R	A	S						
H. sapiens	P	I	N	D	-	I	S	Q	G	R	R	D	A						
A. thalina	P	F	P	D	-	D	G	M	R	L	R	D	S						
S. pombe	P	L	E	N	D	N	A	M	S	N	Q	G	A						

S. cerevisiae (347)	Q	D	R	
H. sapiens	G	F		
A. thalina	G	E		
S. pombe	G	D		
C. elegans	E	D		
T. brucei	T	E	A	

Figure 2

A)



B)

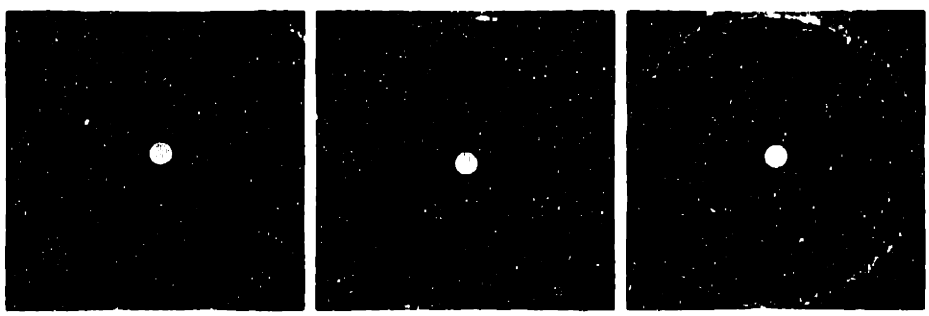


Figure 3

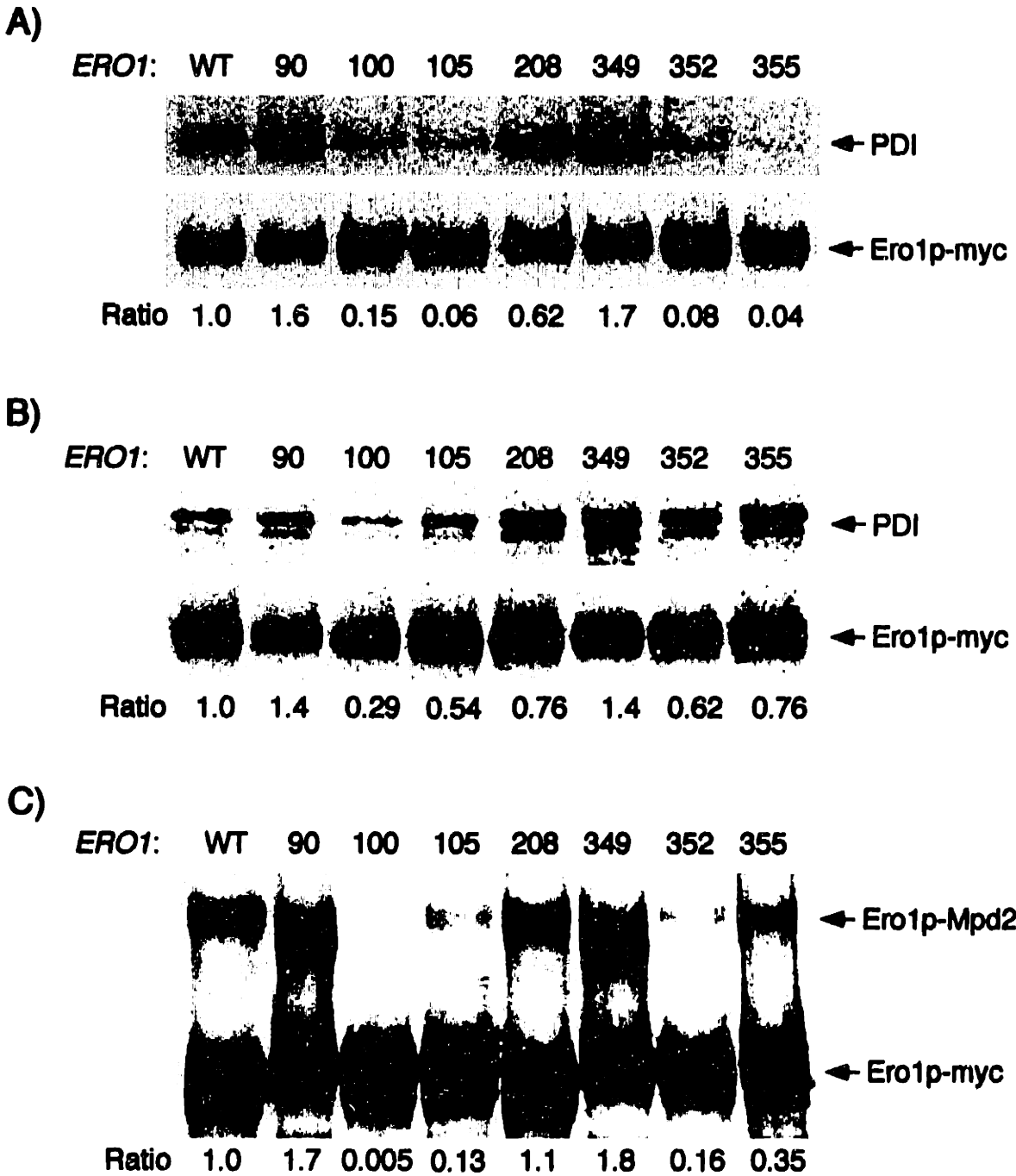

A)
ERO1: WT 90 100 105 208 349 352 355
PDI
Ero1p-myc
Ratio 1.0 1.6 0.15 0.06 0.62 1.7 0.08 0.04
B)
ERO1: WT 90 100 105 208 349 352 355
PDI
Ero1p-myc
Ratio 1.0 1.4 0.29 0.54 0.76 1.4 0.62 0.76
C)
ERO1: WT 90 100 105 208 349 352 355
Ero1p-Mpd2p
Ero1p-myc
Ratio 1.0 1.7 0.005 0.13 1.1 1.8 0.16 0.35

Figure 4

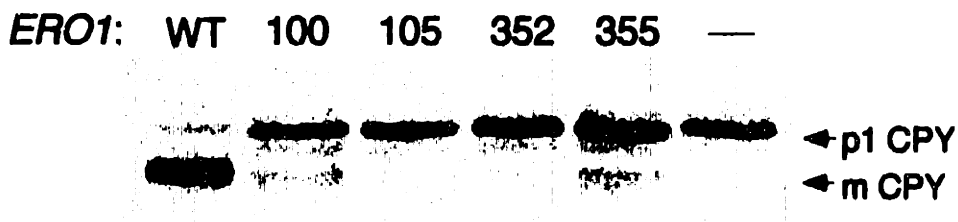


Figure 5

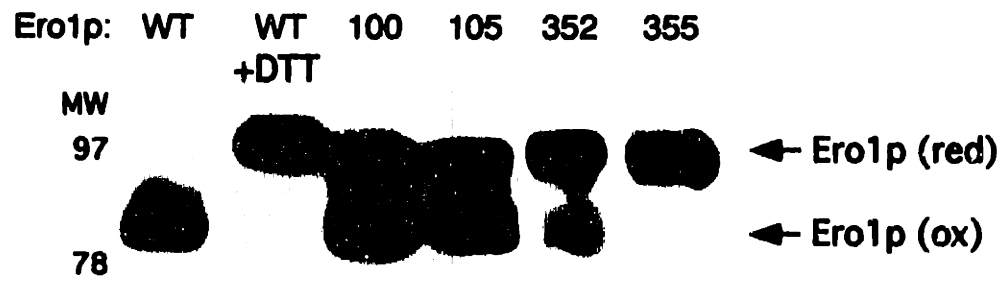


TABLE 1

Yeast Strains

Strain	Genotype	Source
CKY8	*MATα ura3-52 leu2-3,112*	Kaiser Lab Collection
CKY10	*MATa ura3-52 leu2-3,112*	"
CKY263	*MATa GAL2 ura3-52 leu2-3,112*	"
CKY558	*MATa ero1-1 ura3-52 leu2-3,112 ade2*	"
CKY559	*MATα ero1-1 ura3-52 leu2-3,112*	"
CKY561	*MATa ire1-Δ::URA3 ura3-52 leu2-3,112*	"
CKY598	*MATa GAL2 ero1-1 ura3-52 leu2-3,112*	"
CFY599	*MATa/α ero1-Δ-500::LEU2/ERO1 leu2-3,112/leu2-3,112 ura3-52/ura3-52*	This study
CKY600	*MATα ero1-Δ-500::LEU2 ura3-52 leu2-3,112* [pAF82]	"
CKY601	*MATα ero1-Δ-500::LEU2 ura3-52 leu2-3,112* [pAF98]	"
CKY602	*MATα ero1-Δ-500::LEU2 ura3-52 leu2-3,112* [pAF131]	"
CKY603	*MATα ero1-Δ-500::LEU2 ura3-52 leu2-3,112* [pAF99]	"
CKY604	*MATα ero1-Δ-500::LEU2 ura3-52 leu2-3,112* [pAF120]	"
CKY605	*MATα ire1-Δ::URA3 ero1-1 ura3-52 leu2-3,112*	"

Appendix One:

High-Copy Suppressors of *ero1-1*

OVERPRODUCTION OF A POTENTIAL ER GLUTAREDOXIN SUPPRESSES LOSS-OF-FUNCTION MUTATIONS IN *ERO1*.

To isolate additional components of the ER protein oxidizing machinery that may function in conjunction with *ERO1*, we undertook a screen for high-copy suppressors of the conditional *ero1-1* mutant. Six clones were isolated from a yeast cDNA library under *GAL1* promoter control (Liu et al., 1992) that restored growth to *ero1-1* cells at 36°C and ameliorated the sensitivity of *ero1-1* cells to 2 mM DTT at 30°C. Two of these clones proved identical and specified an as yet uncharacterized ORF, YBR014c (Cherry et al., 1997), which is presently referred to as *EGX1* in our laboratory (F. Åslund, personal communication). P_{GAL1}-*EGX1* also rescued the inviability of cells with a complete chromosomal deletion (*ero1-D1-500::LEU2*) of *ERO1*. Although the suppressed strain grew extremely slowly, P_{GAL1}-*EGX1* nevertheless thus bypasses the need for *ERO1* for cell viability.

The predicted product of *EGX1* displays homology to glutaredoxins, ubiquitous GSH-dependent thiol-disulfide oxidoreductases commonly found in the cytosol (Holmgren, 1989). The product of *EGX1*, however, contains a potential signal sequence, suggestive of ER localization. Only one potential secretory glutaredoxin has previously been identified, from a protozoan parasite (Ebel et al., 1997). The predicted homology between Egx1p and glutaredoxins includes residues composing the binding pocket for GSH and the redox-active site CPYC (Bushweller et al., 1994). Egx1p contains the active-site sequence CPYS, but the protein would nevertheless be predicted to possess activity as a glutaredoxin; an E. coli Grx1 mutant with an identical active-site displays activity as an oxidoreductase towards GSH-mixed disulfides in vitro (Bushweller et al., 1992, Lundström-Lung and Holmgren, 1995).

The suppression of *ero1-1* by P_{GAL1}-*EGX1* appears to depend upon intracellular glutathione, but not upon molecular oxygen. In vitro, glutaredoxin can enhance the catalytic effectiveness of PDI in the oxidative refolding of RNase A,

apparently by facilitating the formation and reduction of glutathione-RNase mixed-disulfides at early stages of the folding reaction. Glutaredoxin catalyzes the formation of these mixed-disulfides independently of PDI, and PDI facilitates their conversion to native RNase A (Ruoppolo et al., 1997, Lundström-Lung and Holmgren, 1995). It is not known whether PDI is required for suppression of *ero1-1* by P_{GAL1}-*EGX1*. However, based on the activity of glutaredoxins in vitro, it seems plausible that overproduced Egx1p could facilitate protein oxidation in the ER by driving the formation of substrate-glutathione mixed-disulfides. This mechanism would, however, not explain how Egx1p generates an alternative source of oxidizing activity, compensating for the complete loss of *ERO1*. It therefore also seems possible that Egx1p may suppress *ero1-1* by mobilizing alternative sources of oxidizing equivalents in the ER through an oxidoreductase activity towards GSH-small molecule mixed-disulfides.

Two additional clones (pAF100 and pAF101) were isolated that fully rescued the phenotypes associated with *ero1-1* and corresponded to P_{GAL1}-*ERO1*. A third clone (pAF103) that partially suppressed the *ero1-1* mutation corresponded to P_{GAL1}-*MPD2* (see Chapter 2, Tachikawa et al., 1992).

OVEREXPRESSION OF *FAD1* SUPPRESSES THE CONDITIONAL *ero1-1* MUTATION.

In a parallel screen of a high-copy yeast genomic library, three independent clones were isolated that restored growth to *ero1-1* cells at 36°C. One clone (pAF106) corresponded to *2μ ERO1*. The remaining clones (pAF108 and pAF109) contained overlapping genomic inserts, and their rescuing activity corresponded to *FAD1* (Flavin Adenine Dinucleotide Synthetase 1). This gene encodes the cytosolic enzyme responsible for adenylating flavin mononucleotide (FMN) during biosynthesis of FAD (Wu et al., 1995). *FAD1* overexpression does not appear to bypass *ERO1*, since *2μ FAD1* failed to rescue the inviability of *ero1-Δ1* spores. The mechanism of *ero1-1* suppression by *FAD1* remains to be explored. However, FAD

is known to serve as a cofactor in the NADPH-dependent reduction of catalytic disulfides in enzymes such as thioredoxin reductase and glutathione reductase, raising the intriguing possibility that a flavoenzyme may also serve as an intermediate in one pathway for re-oxidation of Ero1p. If so, overexpression of *FAD1* may suppress *ero1-1* by facilitating re-oxidation of the corresponding mutant of Ero1p.

METHODS

Yeast strains were grown and manipulated using standard techniques (Kaiser et al., 1994). In each screen 7,500 to 15,000 transformants of CKY559 (*ero1-1*) were selected on SC lacking uracil. For the P_{GAL1}-cDNA library, transformants were replicated to YEP Raf/Gal or the same medium containing 2 mM DTT and incubated at either 36°C or 30°C, respectively. To allow for induction of the *GAL1* promoter, plates were held at room temperature for 4 hours prior to incubation at 36°C. Yep24 library transformants were handled similarly, but replicated to YPD. Viable clones were isolated after 1 to 2 days. Suppressed strains were grown on media containing 5-FOA (fluroorotic acid) to allow for verification that loss of the plasmid correlated with loss of the suppressed phenotype. Potential suppressors were also re-transformed into CKY559 and retested for the ability to confer a suppressed phenotype. In the P_{GAL1}-cDNA library screen, re-transformed strains were examined on both YEP Raf/Gal and YPD plates as another means of verifying the dependency of the suppressed phenotype on a locus regulated by the *GAL1* promoter. To test for suppression of the inviability associated with a chromosomal deletion of *ERO1*, CKY562 (*ERO1/ero1-Δ::LEU2 ura3-52/ura3-52 leu2,3-112/leu2,3-112*) was transformed with either pAF102 or pAF116 (*2u FAD1 URA3*). After sporulation, the rescue of *ero1-Δ::LEU2* cells was indicated by the presence of tetrads with more than 2 viable spores, where 1 or 2 viable spores were Leu$^+$. The *ero1-Δ::LEU2* spores hosting pAF102 grew as microcolonies after 5-10 days. To test for growth under anaerobic conditions, plates were incubated in the BRL GasPak system at 30°C.

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Appendix Two:

Tools for the Purification of Ero1p

PREFACE

Tools for the purification of Ero1p outlined in this chapter were developed by A. R. Frand, with the help of technical assistance from Peter Chivers and equipment from the laboratories of Robert Sauer and Graham Walker. Assays for oxidoreductase activity were conducted by J. Cuozzo.

Methods for the purification of Ero1p from yeast were developed in the hopes of obtaining analytical quantities of the protein, in part for use in biochemical assays for oxidoreductase activity. The observation that Ero1p is glycosylated and tightly associated with the ER membrane influenced the decision to isolate Ero1p from yeast, rather than to express the protein in bacteria. A relatively simple assay for oxidoreductase activity monitors an increase in light-scattering associated with the reduction of insulin by DTT. Although fractions containing Ero1p-myc-6xHis as the principal component lacked detectable activity in this assay (J. Cuozzo, personal communication), the behavior of Ero1p-myc through several purification steps may nevertheless be useful in further isolations. The following observations have therefore been included in this Appendix.

SOLUBILIZATION OF ERO1P-MYC

After preparation of a standard yeast extract, Ero1p-myc sediments entirely in the 13,000xg membrane pellet, whereas soluble proteins remain in the supernatant after centrifugation at 100,000xg. The release of Ero1p-myc from membrane to soluble fractions was assessed following the treatment of cell extracts with a panel of detergents. Ero1p-myc remained in the membrane fraction following treatment of extracts with up to 2% octylglucoside. In contrast, Ero1p-myc was readily solubilized by treatment of extracts with either 0.25% digitonin or 0.1% Triton X-100, when 0.05 OD_{600} units of cells were suspended per 1µl volume. At 100-fold higher concentration, efficient release of Ero1p-myc into the soluble fraction required treatment of extracts with 1% Triton X-100.

ANION AND CATION EXCHANGE CHROMATOGRAPHY OF ERO1P-MYC

Ero1p-myc bound to both anion and cation-exchange chromatography resins at near

neutral pH. Following solubilization of the protein (in 50 mM Tris-HCl pH 7.4, 1% Triton X-100), Ero1p-myc was retained on both HiTrap-Q and HiTrap-S columns (Pharmacia). Ero1p-myc could be eluted from the HiTrap-Q column with 500 mM NaCl, and from the HiTrap-S column with 250 mM NaCl. The behavior of Ero1p-myc-6xHis during FPLC over a MonoQ column (Pharmacia) was assessed further. In this case, the elution fraction containing the peak of Ero1p-myc-6xHis corresponded to 380 mM NaCl.

AFFINITY CHROMATOGRAPHY OF ERO1P-MYC-6XHIS

To allow for purification of Ero1p by affinity chromatography on Ni-NTA resin, a functional version of Ero1p-myc was created containing six histidine residues at the C-terminus. Ero1p-myc-6xHis (solubilized in 50 mM Tris-HCl pH 7.4, 100 mM NaCl, 1% Triton X-100, and 5% glycerol) bound efficiently to an Ni-NTA column, and was retained on the column through washes containing 10 mM imidiazole. Ero1p-myc-6xHis could be eluted with 0.5 M imidiazole.

Following a two-step purification protocol involving anion-exchange chromatography on a MonoQ column and affinity chromatography on Ni-NTA resin, fractions were obtained containing Ero1p-myc-6xHis as the principal protein component. By this protocol, approximately 4ug of Erop-myc-6xHis were isolated per OD_{600} unit of yeast extract. Unfortunately, it is not possible to report a fold-enrichment for these purification steps, since a biochemical assay for Ero1p activity has yet to be developed. The possibility exists that affinity chromatography on the Ni-NTA resin inactivates Ero1p, since oxidoreductase activity detectable in peak fractions from the MonoQ column was not recovered following affinity chromatography (J. Cuozzo, personal communication). If so, it may be necessary to develop a purification protocol relying on ion-exchange chromatography in addition to other steps.

METHODS

The myc epitope was appended to P_{GAL1}-*ERO1* by cloning a 0.6 kb HindIII-SacII fragment from pAF82 (Frand and Kaiser, 1998) into pAF100 (*CEN URA3* P_{GAL1}-*ERO1*), thereby generating pAF112. The sequence 5' CAC CAC CAC CAC CAC CAC 3' , specifying the 6xhis tag, was then inserted in frame at the C-terminus of P_{GAL1}-*ERO1-myc* by PCR-based mutagenesis on pAF112, creating pAF121. Expression of either P_{GAL1}-*ERO1-myc* or P_{GAL1}-*ERO1-myc-6xhis* rescued the growth of *ero1-1* cells at high temperature, demonstrating the each allele specified a functional version of Ero1p. For purification of Ero1p, these alleles were expressed in CKY474 (*GAL2 pep4Δ:: ura3-52, leu2-3,112*). Cells were grown selectively overnight and then cultured in YEP Raf/Gal to an optical density (OD_{600}) not exceeding 2.0. Approximately 20,000 OD_{600} units of cells were collected by centrifugation and resuspended in lysis buffer (50 mM Tris-HCl pH 7.4, 100 mM NaCl, 0.2 M Sorbitol, and 1ug/ml final each of PMSF, aprotinin, leupeptin, and pepstatin, all Boerhinger Manheim). Cells were disrupted by vigorous agitation with glass beads at 4°C, and a membrane fraction collected from the lysate by centrifugation at 13,000xg for 30 min at 4°C. The resulting pellets were resuspended in at least four volumes of lysis buffer containing 1% Triton X-100 and 5% glycerol. Soluble material was then collected following centrifugation at 100,000xg for 40 min at 4C (55,000 rpm in a Beckman ultracentrifuge). Samples were brought to 100 mM NaCl prior to affinity chromatography on Ni-NTA resin (Qiagen). For a preparation of this size, a 3 ml bed volume was employed, and the column was washed with a 30 ml volume of 10 mM imidiazole. Ero1p-myc-6xhis was eluted with 5 ml of 0.5 M imidiazole. When necessary, NaCl was removed prior to anion exchange chromatography by dialysis of the sample against 2 liters of lysis buffer lacking NaCl for 3 hr at 4°C. Approximately 5 ml samples corresponding to 10,000 to 20,000 OD_{600} units of cell extract were loaded on a MonoQ column (Pharmacia) and Ero1p diluted with a linear gradient of 0 to 0.7 M NaCl in lysis buffer. Following resolution of all fractions by SDS-PAGE, Ero1p-myc was detected either by immunoblotting with anti-myc as primary antibody (9E10, Covance) and

HRP-conjugated anti-mouse IgG as secondary antibody (Amersham), or by staining with Coomaisse Brilliant Blue. The protein content of various fractions was quantified using the BioRad DC protein assay.

Appendix Three:

Sequence of Ero1p from Yeast

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  1 MRLRTAIATL CLTAFTSATS NNSYIATDQT QNAFNDTHFC
 41 KVDRNDHVSP SCNVTFNELN AINENIRDDL SALLKSDFFK
 81 YFRLDLYKQC SFWDANDGLC LNRACSVDVV EDWDTLPEYW
121 QPEILGSFNN DTMKEADDSD DECKFLDQLC QTSKKPVDIE
161 DTINYCDVND FNGKNAVLID LTANPERFTG YGGKQAGQIW
201 STIYQDNCFT IGETGESLAK DAFYRLVSGF HASIGTHLSK
241 EYLNTKTGKW EPNLDLFMAR IGNFPDRVTN MYFNYAVVAK
281 ALWKIQPYLP EFSFCDLVNK EIKNKMDNVI SQLDTKIFNE
321 DLVFANDLSL TLKDEFRSRF KNVTKIMDCV QCDRCRLWGK
361 IQTTGYATAL KILFEINDAD EFTKQHIVGK LTKYELIALL
401 QTFGRLSESI ESVNMFEKMY GKRLNGSENR LSSFFQNNFF
441 NILKEAGKSI RYTIENINST KEGKKKTNNS QSHVFDDLKM
481 PKAEIVPRPS NGTVNKWKKA WNTEVNNVLE AFRFIYRSYL
521 DLPRNIWELS LMKVYKFWNK FIGVADYVSE ETREPISYKL
561 DIQ
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Sequence predicted from translation of Saccharomyces cerevisiae open reading frame YML130c (Cherry et al., 1997).

Chapter Five:

Future Directions for Exploring Ero1p Function and Oxidative Protein Folding in the Endoplasmic Reticulum

This thesis describes the isolation of a novel gene, *ERO1*, required for the oxidation of protein thiols in the endoplasmic reticulum. Oxidizing equivalents flow from Ero1p to PDI to secretory proteins in a series of direct thiol-disulfide exchange reactions. This pathway obviates the need for oxidized glutathione for protein disulfide bond formation in vivo. The oxidizing activity of Ero1p has been traced to two pairs of conserved cysteines likely to form redox-active disulfides bonds in Ero1p.

Isolation of *ERO1* opens numerous avenues for further investigation into the mechanisms underlying ER redox homeostasis and oxidative protein folding in vivo. Perhaps the most important and intriguing question that remains concerning the function of Ero1p is the identity of its physiologic oxidant(s). The tight association of Ero1p with membranes may allow oxidation of Ero1p to be coupled to electron transport chains mediating fatty acid synthesis in the ER membrane. Partially redundant pathways may contribute to Ero1p oxidation under different growth conditions. The net flux of electrons from protein disulfide bond formation may also be minimal relative to the flux of electrons from other metabolic pathways in the cell. Consequently, the development of an in vitro assay for Ero1p activity may facilitate the identification of oxidants for the enzyme, just as the development of an assay for DsbB activity expedited the isolation of cytochrome bd oxidase as an electron acceptor for the enzyme in vitro (Bader et al., 1999).

A further question concerns the nature of Ero1p-independent pathways for oxidative protein folding that have been revealed by the isolation of bypass suppressors of *ERO1*. For example, mutation of *GSH1*, a gene required for glutathione biosynthesis, can restore viability to cells with a chromosomal deletion of *ERO1* (J. Cuozzo, personal communication). This observation indicates that a background oxidizing activity exists in the ER that can support the oxidation of essential secretory proteins when the reducing power of glutathione is removed. The nature of this relatively inefficient oxidizing activity remains unclear, but the pathway may depend upon molecular oxygen as a final electron acceptor, since

gsh1Δ ero1-Δ1-500 cells are inviable under anaerobic growth conditions. It is not known if this pathway depends upon PDI. Theoretically, additional components of this pathway could be identified through screens for suppressors or enhancers of the poor growth of *gsh1Δ ero1-Δ1-500* cells.

The precise mechanism of disulfide bond isomerization in the ER also remains unclear. In E. coli, the isomerase activity of DsbC requires a net influx of reducing equivalents from the cytoplasmic membrane protein DsbD (Rietsch et al., 1997). If an analogous pathway operates in the ER, then glutathione, or an as yet uncharacterized ER oxidoreductase, could be responsible for maintaining a portion of PDI in the reduced form, enabling PDI to function as an isomerase in vivo. Theoretically, glutathione could also reduce unstable or non-native protein disulfide bonds directly.

Analysis of disulfide bond isomerization in the yeast ER would be greatly facilitated by the availability of a secretory marker protein with multiple disulfide bonds. The fate of such a marker protein in various mutant backgrounds could be compared to the fate of a secretory protein with only a single structural disulfide. In E. coli, the activity of heterologous mouse urokinase, which possesses 12 native disulfide bonds, has proven a sensitive assay for the function of the periplasmic reducing pathway (Rietsch et al., 1996, Rietsch et al., 1997). Theoretically, this marker protein could also be expressed in the ER, and genes required for disulfide isomerization could be isolated as mutations specifically disrupting urokinase folding. The oxidative folding of urokinase could also be assessed in cells deficient in glutathione, PDI, or any of the PDI-homologs resident in the ER.

With the identification of the central components required for protein oxidation in vivo, it now seems reasonable to develop biochemical assays recapitulating steps of this process in vitro. The preparation of analytic quantities of active Ero1p would allow for determination of the redox potentials of the active-sites of the enzyme, possibly through equilibration with glutathione. An assay for the oxidation of Ero1p or the Ero1p-catalyzed oxidation of PDI may, as discussed above, greatly expedite the search for physiologic oxidants of Ero1p. An assay for the catalysis of PDI

oxidation by Ero1p would further allow for determination of the enzymatic properties of Ero1p, including its K_m for PDI. Unfortunately, the tight association of Ero1p with membranes may limit the development of such biochemical assays.

In chapter 4, a model for Ero1p function is proposed wherein the Cys100-Cys105 disulfide of Ero1p specifically engages in thiol-disulfide exchange with thioredoxin-like oxidoreductases in the ER, while the Cys352-Cys355 disulfide serves to re-oxidize the Cys100-Cys105 cysteine pair, possibly through an intramolecular thiol-disulfide exchange reaction. A definitive identification of the cysteine residue in Ero1p that interacts with the active-site cysteines of PDI is needed to confirm this model. One approach to this problem may involve the formation, in vitro, of sufficient quantities of PDI-Ero1p mixed-disulfides for analysis by mass spectrometry. More robust evidence of intramolecular thiol-disulfide exchange is also required to confirm this model. Here, one approach involves documenting intermolecular disulfide transfer between the active-sites of co-overproduced cysteine to alanine substitution mutants of Ero1p. Preliminary evidence suggests that intermolecular disulfide transfer can occur between the Cys352-Cys355 active-site of one Ero1p molecule and the Cys100-Cys105 active-site of another when an intramolecular acceptor site for the Cys352-Cys355 disulfide is not available. Mixed-disulfides have been detected between CA352 Ero1p-myc and a functional version of CA100 Ero1p tagged with the HA epitope. Further, co-overexpression of *ero1-A105-myc* and *ero1-A352-myc* has been observed to rescue the temperature-sensitive growth defect of *ero1-1* cells, even though overexpression of either mutant alone fails to rescue this strain (data not shown). These observations suggests that the active-sites in two different Ero1p molecules can work together to complete the function of Ero1p. The extent to which intermolecular thiol-disulfide exchange occurs between wild-type Ero1p molecules remains to be explored.

The isolation of *ERO1* provides an entry point for the genetic analysis of oxidative protein folding in the ER. Analysis of Ero1p function may reveal the link between protein oxidation and general cellular metabolism, and the mechanisms

underlying ER redox homeostasis in yeast may function throughout eukaryotes.

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