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

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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 nonnative 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 endoplasmic reticulum (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.

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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 nonnative 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* (Tachibana and Stevens, 1992; Tachikawa et al., 1995, 1997). 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.

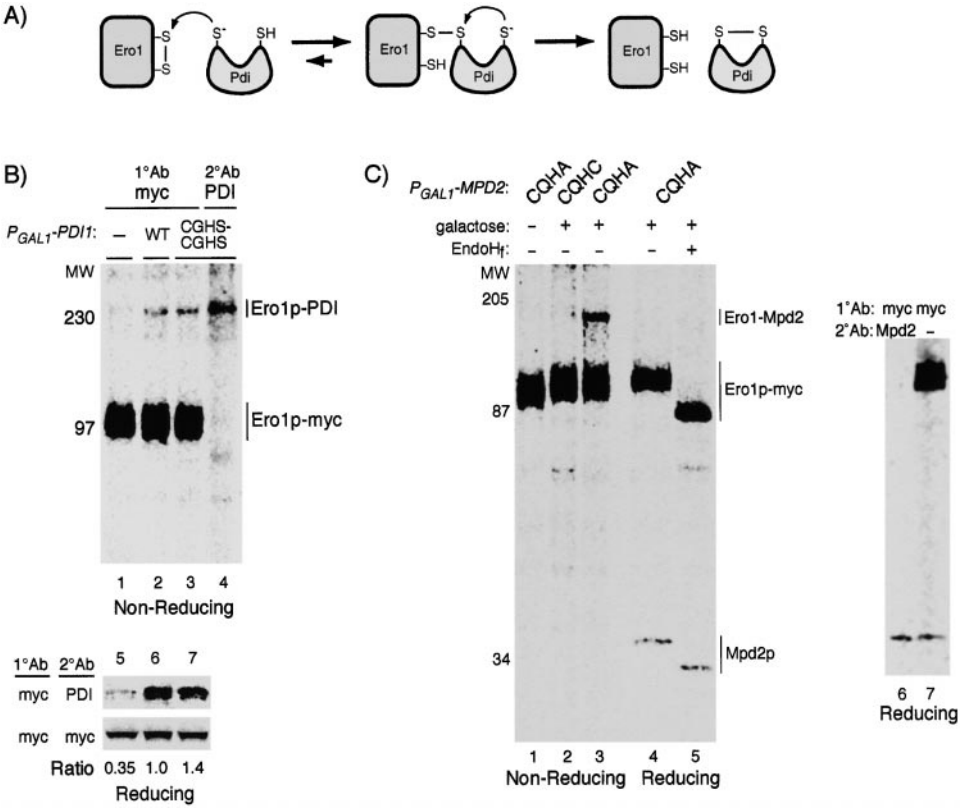

Figure 1. Trapping PDI–Ero1p and Mpd2p–Ero1p Mixed Disulfides

(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 and 6) or CGHS-CGHS PDI (lanes 3 and 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 nonreducing conditions. In addition to free Ero1p–myc, 230 kDa complexes were detected (lanes 2 and 3) that were reimmunoprecipitated with anti-PDI antibody under nonreducing conditions (lane 4, 5× loading). The anti-myc immunoprecipitates were reduced with DTT, divided, and reimmunoprecipitated with anti-PDI or anti-myc antibody (lanes 5–7), allowing quantitation of the PDI trapped in mixed disulfides with Ero1p–myc (see Experimental Procedures). Anti-PDI samples were loaded 6× 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 and 5) in addition to pCT37 (P_{GAL1}*-PDI1*, lanes 2 and 6) or pAF132 (P_{GAL1}*-pdi1-CGHS-CGHS*, lanes 3, 4, and 7).

(C) Primary anti-myc immunoprecipitates were prepared as in (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 deglycosylation with $EndoH_f$ (lanes 4 and 5). In a parallel experiment, this 35 kDa protein (lane 7) was reimmunoprecipitated by anti-Mpd2p antibody (lane 6, 3× loading). Strains were derived from CKY263 and hosted pAF89 in addition to pAF123 (P_{GAL1}*-mpd2-CQHA*, lanes 1 and 3–7) or pAF103 (P_{GAL1}*-MPD2*, lane 2). In lane 1, expression of P_{GAL1}*-mpd2-CQHA* was repressed by growth on glucose.

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 nonreducing but denaturing conditions. In addition to free Ero1p–myc, high–molecular weight complexes could be detected (Figure 1B, lanes 2 and 3). These complexes were shown to represent PDI–Ero1p mixed disulfides by reimmunoprecipitation with anti-PDI under nonreducing 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 nonreducing 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 reimmunoprecipitation 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 nonreducing 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 and 2). Further evidence that these complexes represented CQHA Mpd2p–Ero1p mixed disulfides was derived from the observation that a 35 kDa glycoprotein was released when the complexes were reduced by DTT (Figure 1C, lanes 4 and 5). Consistent with assignment of this protein as Mpd2p, a comigrating protein could be reimmunoprecipitated by anti-Mpd2p antibody following reduction of the mixed disulfides (Figure 1C, lanes 6 and 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 reimmunoprecipitated with anti-CPY antibody. There was no evidence of mixed disulfides 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 nonnative 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

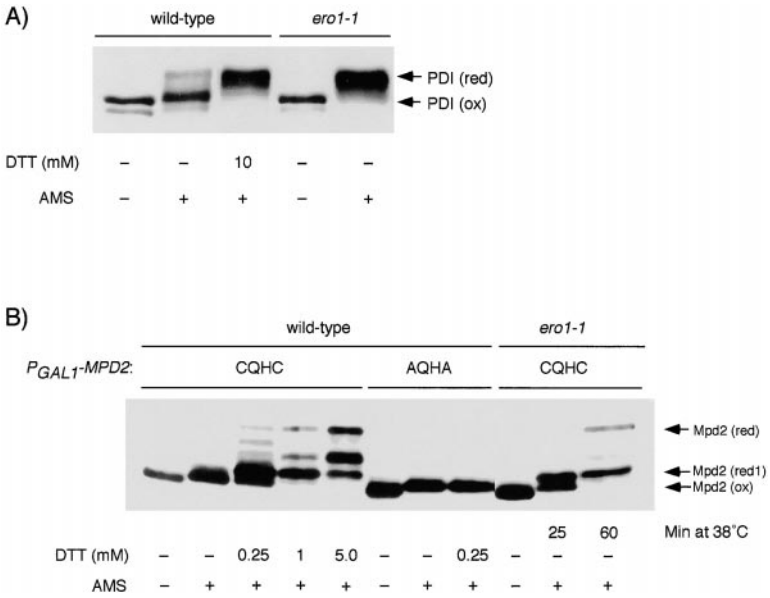

Figure 2. *ERO1* Maintains PDI and Mpd2 in Oxidized Form In Vivo

(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.

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 hr, 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 nonreducing but denaturing conditions. At this stage, discrete complexes representing mixed disulfides between endogenous PDI and any single ER substrate were not readily detected after nonreducing 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 reimmunoprecipitated with anti-CPY antibody. Approximately 7% of the total p1 CPY was reimmunoprecipitated 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 reimmunoprecipitated 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 nonreducing SDS-PAGE with reduced p1 CPY (LaMantia and Lennarz, 1993). However, since misoxidized or partially oxidized p1 CPY may comigrate with fully reduced p1 CPY during nonreducing 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 ten 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 hr 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

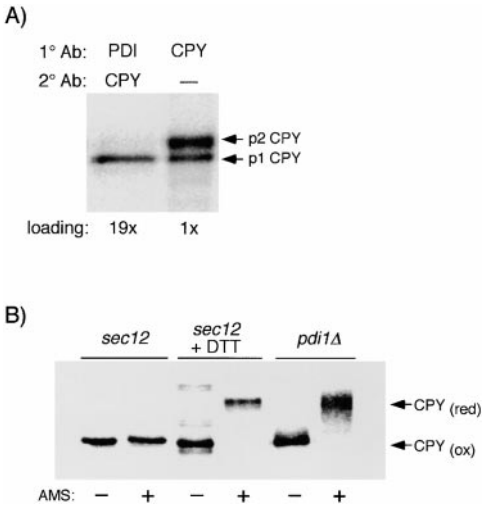

Figure 3. PDI Functions as an Oxidase In Vivo

(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 nonreducing but denaturing conditions. Reduction of the sample with DTT followed by reimmunoprecipitation 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 *pdi1*Δ strain expressing P_{GAL1}-*PDI1* (CKY564) was grown in 2% glucose for 18 hr. 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.

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 nonreducing SDS-PAGE (Figure

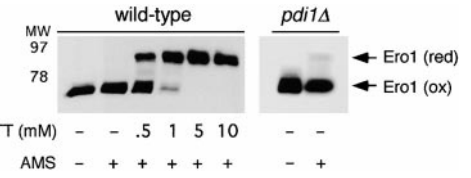

Figure 4. Ero1p Is Oxidized In Vivo

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

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 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 in which 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* (LaMantia and Lennarz, 1993; Frand and Kaiser, 1998; Pollard et al., 1998). 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

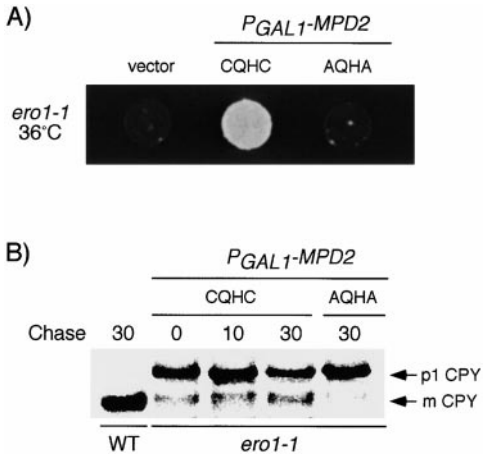

Figure 5. *MPD2* Facilitates Oxidative Protein Folding in the ER

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 4×10^7 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 were 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.

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 reoxidation 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 (Laboissière et al., 1995; Walker et al., 1996). CGHS-CGHS PDI can nevertheless rescue the inviability of cells with a chromosomal deletion of *PDI1* (LaMantia and Lennarz, 1993; Laboissière et al., 1995). Overexpression of *EUG1*, which encodes a PDI homolog with CLHS and CIHS active sites, can also restore growth to PDI-deficient cells (Tachibana

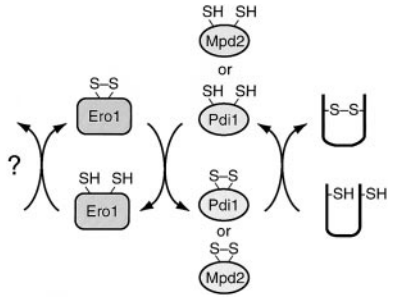

Figure 6. Model for Protein Disulfide Bond Formation in the ER Lumen

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.

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 nonnative 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 *pdi1*Δ 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, *pdi1*Δ 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 *pdi1*Δ 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 (Chivers et al., 1997; Holst et al., 1997). Further, *E. coli* thioredoxin localized to the yeast ER can rescue the inviability of *pdi1*Δ cells only after the CXXC motif of the enzyme is modified to have a higher redox potential and lower pK_a (Chivers et al., 1996, 1997). These changes enable thioredoxin to serve as a more efficient isomerase, and oxidase, in vivo.

Table 1. Yeast Strains

Strain	Genotype	Source
CKY10	*MAT***a** *ura3-52 leu2-3, 112*	Kaiser lab collection
CKY39	*MAT*α *sec12-4 ura3-52 his4-619*	Kaiser lab collection
CKY263	*MAT***a** *ura3-52 leu2-3, 112 GAL2*	Kaiser lab collection
CKY559	*MAT*α *ero1-1 ura3-52 leu2-3, 112*	Kaiser lab collection
CKY598	*MAT***a** *ero1-1 ura3-52 leu2-3, 112 GAL2*	Kaiser lab collection
CKY612	*MAT***a** *mpd2-*Δ*::HIS3 ura3-52 leu2-3, 112 his3-*Δ*200*	Kaiser lab collection
CKY564	*MAT*α *pdi1-*Δ*::HIS3 ura3-1 leu2-3, 112 his3-11, 15 trp1-1 ade2-1 can1-100* [pCT37]	Tom Stevens

PDI was first isolated from rat liver microsomes as an activity catalyzing the reactivation 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 nonnative 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 reoxidation of PDI by GSSG in vivo.

Here we present data indicating that the oxidase activity of PDI in vivo is coupled to reoxidation 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 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 reoxidize 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 reoxidized 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 nonnative 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 (Bader et al., 1999; 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 2×10^7 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 et al., 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 *2*μ *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 nonreducing sample buffer (80 mM Tris–HCl [pH 6.8], 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 (1 μg/ml each pepstatin, aprotinin, and leupeptin) were included when trapping PDI. Samples were adjusted to pH 6.8 by gradual addition of 1 M 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 reimmunoprecipitated with anti-PDI under nonreducing conditions. Otherwise, 1/5 of each sample was saved for analysis by nonreducing SDS-PAGE, and the rest boiled for 3 min in sample buffer with 100 mM DTT. Of each sample, 1/5 was reimmunoprecipitated with 9E10 antibody while 3/5 was reimmunoprecipitated with anti-PDI antibody (kindly provided by Tom Stevens). As a control, supernatants from this last step were immunoprecipitated with anti-CPY antibody. To quantitate 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 reimmunoprecipitated PDI (per OD_{600} U of extract) to that of reimmunoprecipitated 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 nonreducing conditions. A portion of the sample was saved for analysis by nonreducing SDS-PAGE, and the rest boiled for 3 min in sample buffer with 100 mM DTT. A sample corresponding to 28 OD_{600} U of extract was reimmunoprecipitated with anti-CPY antibody (kindly provided by Hidde Ploegh). To quantitate 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 nonreducing 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 deglycosylated by 4-fold dilution into 50 mM sodium citrate (pH 5.5) containing 100 U of $EndoH_f$ and incubated at 37°C for 2 hr. Samples were resolved by nonreducing (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 (*pdi1*Δ 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 hr. 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 nonreducing sample buffer with or without 25 mM AMS. Samples were brought to pH 6.8, incubated on ice for 15 min, incubated 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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