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Biochemical basis of oxidative protein folding in the endoplasmic reticulum

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Biochemical Basis of Oxidative Protein Folding in the Endoplasmic Reticulum

by

Benjamin Peng-Chu Tu

DISSERTATION

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GRADUATE DIVISION

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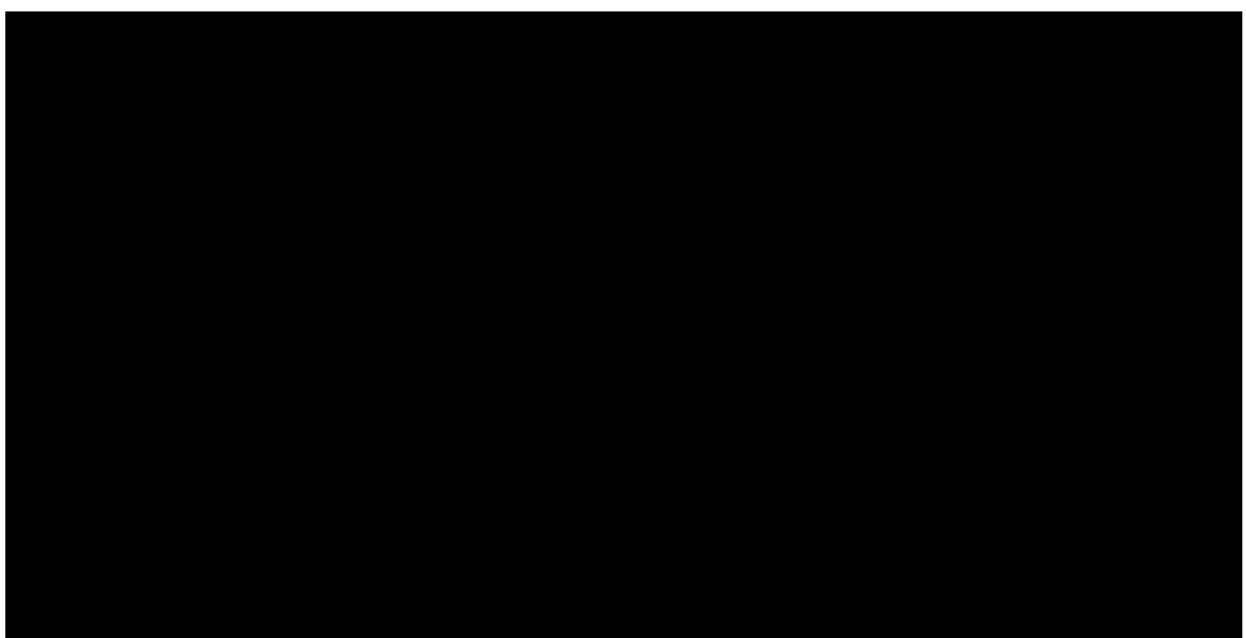

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for my family

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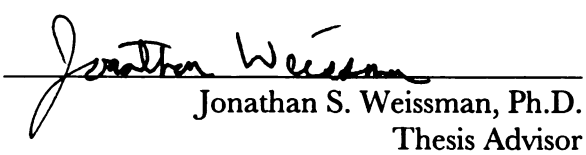
ABSTRACT

This work has focused on understanding how disulfide bonds are formed in folding proteins as they travel through the secretory pathway. The endoplasmic reticulum (ER) supports the formation of disulfide bonds in newly translocated proteins through a mechanism dependent on the protein Ero1p (ER oxidoreductin 1). I developed a purification procedure to purify Ero1p from yeast microsomes and determined that it bound flavin adenine dinucleotide (FAD). With this finding, I was able to reconstitute efficient oxidative folding in vitro using purified Ero1p, protein disulfide isomerase (PDI), and FAD. I demonstrated that Ero1p is a specific, FAD-dependent oxidase of PDI, which subsequently introduces disulfide bonds into folding proteins. The catalysis of disulfide formation by Ero1p and PDI proceeds through a series of disulfide exchange reactions and is not dependent on glutathione. FAD-bound Ero1p then uses molecular oxygen as its terminal electron acceptor to reoxidize itself. In parallel, by depleting various redox molecules from yeast and examining the consequences on disulfide formation, we were able to confirm that oxidative folding in vivo is highly dependent on cellular levels of FAD.

Ero1p-catalyzed disulfide formation proceeds rapidly even in the presence of reduced glutathione. This kinetic shuttling of oxidizing equivalents could allow the ER to support rapid disulfide formation while maintaining the ability to reduce and rearrange incorrect disulfides. Glutathione is not the primary source of oxidizing equivalents, but functions instead as a net reductant. The high oxidized glutathione content in the ER is an indirect consequence of Ero1p activity.

Ero1p is the first described flavoprotein localized entirely within the ER, defining a novel role for FAD within the ER lumen. I have also established the presence of a robust transport system that imports FAD into the ER. The Ero1p oxidation system is highly sensitive to physiological levels of free FAD. High levels of free FAD stimulate the Ero1p-driven oxidation cycle, while low levels hinder the cycle. Overall, I have determined the biochemical basis of oxidative protein folding in the endoplasmic reticulum, and my results suggest that controlling the levels of FAD available to Ero1p could be a means to regulate oxidative folding according to a cell's nutritional or metabolic state.



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CONTRIBUTIONS

Portions of the text and figures presented in this thesis are reproduced with permission from material published previously. Chapter 2 "Biochemical Basis of Oxidative Protein Folding in the Endoplasmic Reticulum" was published in November 2000 in Science, Vol. 290, pp. 1571-1574. The CPY refolding assays and the rescue of *ero1-1* by overexpression of *FAD1* in Figure 1A-1C, and Figures 10 and 11, were the work of Dr. Siew Ho-Schleyer. The stimulation of PDI oxidation by the addition of FAD to microsomes in Figure 1D, and Figures 7 and 8, were the work of Dr. Kevin Travers. Chapter 3 "The FAD and O_2-Dependent Reaction Cycle of Ero1-Mediated Oxidative Protein Folding in the Endoplasmic Reticulum" was published in November 2002 in Molecular Cell, Vol. 10, pp. 983-994. The synthesis of azido-FAD was worked out by Dr. Jonathan S. Weissman. With the exception of those items listed above, the work presented in this thesis was performed by its author, Benjamin P. Tu, under the supervision of Dr. Jonathan S. Weissman.

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CHAPTER 1

Introduction

INTRODUCTION

A brief history of oxidative folding

Proteins that traverse the secretory pathway often depend on disulfide bonds for their maturation and function. These bonds are often crucial for the stability of a final protein structure, and the mispairing of cysteine residues can prevent proteins from attaining their final conformation and lead to misfolding. Classic experiments by Anfinsen provided evidence that disulfide formation is a spontaneous process and that the polypeptide itself is sufficient for achieving the native state in vitro (Anfinsen et al., 1961). However, the slow rate of this spontaneous process relative to that expected for correct disulfide formation in vivo led to the hypothesis that disulfide formation is catalyzed in vivo.

In eukaryotes, this process of oxidative protein folding occurs in the endoplasmic reticulum (ER). The formation of disulfide bonds is an oxidative reaction and must be coupled to the reduction of dedicated electron acceptors. Over the past forty years, a number of different factors have been proposed to contribute to maintaining the oxidized environment of the ER, including preferential secretion of reduced thiols, uptake of oxidized thiols, as well as a variety of different redox enzymes and small molecule oxidants (Ziegler and Poulsen, 1977; Hwang et al., 1992; Carelli et al., 1997; Frand et al., 2000). However, primarily due to a lack of genetic evidence, the physiological relevance of these to oxidative folding has remained unclear. Recently, a combination of genetic and biochemical studies using the yeast *Saccharomyces cerevisiae* have begun to reveal the proteins and mechanisms behind this fundamental protein folding process.

The components of the eukaryotic oxidative folding machinery

Genetic screens in yeast have identified a gene *ERO1* (ER oxidoreductin 1) as an essential component of the oxidative folding machinery (Frand and Kaiser, 1998; Pollard et al., 1998). Mutations in *ERO1* lead to sensitivity to the reductant dithiothreitol (DTT) and the accumulation of folding proteins in a reduced form in the ER (Frand and Kaiser, 1998; Pollard et al., 1998). In humans, there are two ERO1 isoforms, hERO1-Lα and hERO1-Lβ (Cabibbo et al., 2000; Pagani et al., 2000). Ero1p is a membrane-associated ER-resident protein and possesses seven conserved cysteine residues that may be involved in catalyzing electron transfer (Frand and Kaiser, 1998; Pollard et al., 1998; Frand and Kaiser, 2000). However, Ero1p has no homology to any redox enzymes or other known proteins. The human isoforms lack a C-terminal tail of ~127 amino acids required by the yeast protein for membrane association (Pagani et al., 2001). Membrane association of Ero1p may allow the protein to be retained in the ER and facilitate cotranslational disulfide formation. Consistent with a function in folding in the ER, yeast Ero1p and hERO1-Lβ are induced by the unfolded protein response (UPR) (Pagani et al., 2000; Travers et al., 2000).

Protein disulfide isomerase (PDI) has also been implicated in the formation of disulfide bonds. PDI constitutes approximately 2% of the protein in the ER and contains two thioredoxin-like Cys-Gly-His-Cys (CGHC) active sites (Goldberger et al., 1965; Laboissiere et al., 1995). PDI has been shown to catalyze disulfide bond formation, isomerization, as well as reduction on a wide range of substrates in vitro (Freedman, 1989), but its role in vivo has been somewhat unclear. The finding that CGHS active site mutants of PDI result in sensitivity to DTT provided strong evidence of a role for PDI in

the formation of disulfide bonds in vivo (Holst et al., 1997). This mutant PDI cannot function as an oxidase, but can still catalyze the isomerization of protein disulfides. While these observations suggest that the essential role of PDI is to unscramble non-native disulfide bonds (Laboissiere et al., 1995), the DTT-sensitivity phenotype of this mutant PDI argues that PDI normally plays an important role in catalyzing the formation of disulfide bonds. Furthermore, in *ero1-1* mutants, PDI accumulates in a reduced form, suggesting that Ero1p acts upstream of PDI in a pathway for disulfide formation in the ER (Frand and Kaiser, 1999).

Recently, the sulfhydryl oxidase Erv2p has been proposed to contribute to oxidative folding in the ER (Sevier et al., 2001). Erv2p is a member of the ERV/ALR family of sulfhydryl oxidases (Thorpe et al., 2002) which have been found in many subcellular compartments and can catalyze the formation of disulfides in proteins directly. Although Erv2p can compensate for defects in Ero1p when overexpressed (Sevier et al., 2001), a role for physiological levels of Erv2p in oxidative folding in the ER has yet to be established.

The source of oxidizing potential for the ER

Glutathione is the major redox buffer in eukaryotic cells (Hwang et al., 1992). The ratio of reduced (GSH) to oxidized (GSSG) glutathione is ~100:1 in the cytosol (Hwang et al., 1992), which are highly reducing conditions that disfavor disulfide formation. However, in the ER where disulfide formation occurs, the ratio of GSH:GSSG is much more oxidizing at ~3:1 (Hwang et al., 1992). This abundance of GSSG in the secretory pathway was long thought to be the source of oxidizing

equivalents for disulfide formation (Hwang et al., 1992). However, genetic evidence in yeast has demonstrated that glutathione is dispensable for disulfide formation and instead functions as a net reductant in the ER (Cuozzo and Kaiser, 1999) (see also below).

What then is the source of oxidizing potential for eukaryotic disulfide formation? In bacteria, disulfide formation is coupled to cellular respiration through the oxidation of quinones (Bader et al., 1999). The periplasmic membrane protein DsbB, which plays an analogous role to Ero1p, reduces ubiquinone to reoxidize itself after one round of disulfide formation (Bader et al., 1999; Bader et al., 2000). Ubiquinone then becomes reoxidized by the respiratory chain with molecular oxygen serving as the terminal electron acceptor. A reverse genetic approach was used to identify the source of oxidizing equivalents for eukaryotic disulfide formation (Tu et al., 2000). Unlike prokaryotes, oxidative folding in yeast is not dependent on cellular respiration, as it is not affected by the absence of ubiquinone or heme (Tu et al., 2000). However, depletion of riboflavin from yeast results in a striking defect in oxidative folding (Tu et al., 2000). Furthermore, overexpression of *FAD1*, which converts flavin mononucleotide (FMN) to flavin adenine dinucleotide (FAD), strongly suppresses the temperature sensitivity of an *ero1-1* mutant (Tu et al., 2000). These observations strongly suggested that oxidative folding in eukaryotes is dependent on cellular FAD levels.

Further evidence that oxidative folding in eukaryotes is dependent on flavins came from the discovery that purified Ero1p itself is a novel FAD-binding protein (Tu et al., 2000). In addition to Ero1p, PDI and FAD are the only other components required to reconstitute robust oxidative folding in vitro (Tu et al., 2000). A mutant PDI with CGHA

active sites acts as a dominant inhibitor of this Ero1p-catalyzed reaction, and a mixed disulfide crosslink could be captured between Ero1p and this mutant PDI both in vivo and in vitro (Frand and Kaiser, 1999; Tu et al., 2000). These observations demonstrated that Ero1p oxidizes PDI directly through disulfide exchange in a FAD-dependent reaction (Tu et al., 2000). Ero1p cannot directly oxidize folding substrates and thus relies on PDI as an intermediary in the transfer of oxidizing equivalents to folding proteins (Tu et al., 2000).

Although FAD is crucial for the activity of Ero1p, it is not functioning as the terminal electron acceptor for Ero1p-catalyzed disulfide formation. Each FAD-bound Ero1p molecule can support multiple rounds of PDI oxidation, and an excess of free FAD cannot drive Ero1p-catalyzed disulfide formation under anaerobic conditions (Tu and Weissman, 2002). These observations suggested that molecular oxygen and not FAD is functioning as the terminal electron acceptor. In vitro experiments confirmed that Ero1p-catalyzed disulfide formation is highly compromised under anaerobic conditions, and that Ero1p directly consumes molecular oxygen during its reaction cycle (Tu and Weissman, 2002). The efficient use of molecular oxygen as the terminal electron acceptor by FAD-bound Ero1p could explain how the oxidation of millimolar concentrations of PDI (Gilbert, 1990) is achieved despite FAD concentrations in the low micromolar range (Gliszczynska and Koziolowa, 1998). Ero1p likely uses alternate terminal electron acceptors under anaerobic conditions, though the identity of these acceptors remains unknown.

The role of FAD in oxidative folding

The dependency of oxidative folding on FAD defines a novel role for the versatile redox molecule in the ER lumen. Ero1p is the first described flavoprotein localized entirely within the ER lumen, and the existence of a robust transport system that imports FAD into the ER lumen has been established (Tu and Weissman, 2002). Oxidative folding in vivo is highly sensitive to specifically free cellular FAD levels (Tu and Weissman, 2002). This sensitivity could be recapitulated in vitro using pure proteins, as the activity of Ero1p varied significantly with small deviations from physiological FAD concentrations (Tu and Weissman, 2002). This sensitivity of Ero1p to free FAD levels may provide a means to regulate oxidative folding (see also below).

The role of PDI and its homologs in oxidative folding

Despite its ability to catalyze a wide range of thiol-disulfide reactions, PDI is found predominantly in an oxidized form in vivo (Frand and Kaiser, 1999), implying its main cellular function is to oxidize folding proteins. Interestingly, there are four homologs of PDI (*EUG1, MPD1, MPD2, EPS1*) in yeast and dozens more are found in higher eukaryotes. Preliminary experiments suggest that yeast Ero1p cannot interact with several of the PDI homologs in vitro (Tu, B.P., unpublished data). Furthermore, hERO1-Lα does not appear to oxidize ERp57, a close relative of human PDI (Mezghrani et al., 2001). However, a mixed disulfide crosslink between Ero1p and a CGHA active site mutant of Mpd2p has been immunoprecipitated from yeast (Frand and Kaiser, 1999), but the functional relevance of this interaction remains to be determined.

The inability of Ero1p to interact with several PDI homologs suggests that it is a rather specific oxidant of PDI. The insulation of these homologs from Ero1p activity may allow them to function as dedicated disulfide isomerases or reductases, which require cysteine residues in their thioredoxin-like active sites to be in the reduced form. It now appears that the role of these PDI homologs is dictated by whether they can interact with upstream oxidases or reductases, rather than the redox potential of their active sites. As in the case of the bacterial protein DsbC, dimerization of the protein prevents oxidation by DsbB and allows it to function as a dedicated isomerase. Mutants of DsbC that disrupt dimerization become oxidized by DsbB and can serve the role of the oxidase DsbA (Bader et al., 2001). In addition, DsbC is normally maintained in the reduced form by the periplasmic membrane protein DsbD, which transfers the reducing power of cytosolic thioredoxin to DsbC (Rietsch et al., 1997). Some of the eukaryotic PDI homologs may similarly be kept in a reduced form by an upstream reductase. Thus, despite the high degree of homology between these PDI homologs, it is apparent they have evolved to carry out disparate redox functions, and future work should lend insight into the determinants of function.

The role of glutathione in oxidative folding

Ero1p-catalyzed disulfide formation proceeds independent of glutathione both in vivo and in vitro (Cuozzo and Kaiser, 1999; Tu et al., 2000). What then is the role of glutathione in disulfide formation? Genetic evidence in yeast indicates that glutathione is acting as a net reductant in the ER. In a screen for suppressors of *ero1-1* temperature sensitivity, a deletion of *GSH1*, which is involved in the biosynthesis of glutathione, was found to strongly suppress the *ero1-1* phenotype (Cuozzo and Kaiser, 1999). The

interpretation of this observation is that absence of a glutathione-mediated reductant might allow a compromised *ero1-1* oxidation system to support growth. In strains lacking glutathione, the oxidative folding of CPY proceeds with normal kinetics but is highly sensitive to oxidative stresses, consistent with the role of glutathione as a net reductant (Cuozzo and Kaiser, 1999).

What is the basis for the high GSSG content in the ER? Ero1p cannot directly oxidize GSH to GSSG (Tu et al., 2000). However, in vitro, Ero1p, PDI, and FAD can drive the oxidation of folding substrates even in the presence of reduced glutathione (GSH). Over time, a gradual production of GSSG resulting from GSH-mediated reduction of disulfides in PDI and folding proteins is observed (Tu et al., 2000). Thus, the abundance of GSSG in the ER is likely an indirect consequence of Ero1p activity, and the GSH:GSSG redox buffer in the ER represents an equilibrium between the consequences of Ero1p-mediated oxidative and glutathione-mediated reductive processes. As alluded to earlier, the kinetic shuttling of oxidizing equivalents by Ero1p and PDI that occurs independent of the bulk redox environment could explain how the ER supports rapid disulfide formation while maintaining the ability to reduce or rearrange incorrect disulfides, perhaps through glutathione and certain PDI homologs.

Oxidative folding as a source of cellular oxidative stress

It seems fitting that molecular oxygen can serve as the terminal electron acceptor for disulfide formation in both prokaryotes and eukaryotes. The complete four-electron reduction of molecular oxygen to water is intrinsically difficult, as its reduction intermediates and byproducts such as superoxide and hydrogen peroxide are highly

reactive and damaging to macromolecules. In bacteria, this problem is solved by coupling oxidative folding to the respiratory chain (Bader et al., 2000), which consists of a complex series of membrane electron transfer proteins that reduce molecular oxygen to water. However, since in eukaryotes oxidative folding and respiration are confined to separate organelles, the Ero1p oxidation system has evolved to function independently of respiration and adopted the use of flavin-based redox chemistry (Tu et al., 2000). In contrast to quinones, FAD is a relatively weak oxidant due to a low redox potential, but the ability of FAD-bound Ero1p to rapidly pass electrons directly to O_2 provides the driving force for disulfide formation.

It remains unclear what happens to the molecular oxygen consumed by Ero1p. It does not appear that Ero1p is releasing stoichiometric amounts of hydrogen peroxide per disulfide formed, although small levels of hydrogen peroxide can be detected (~1 per 20 disulfides formed) during its catalysis of disulfide formation (Tu et al., 2000). However, on the cellular level, uncontrolled Ero1p oxidase activity could be a significant source of oxidative stress. Consistent with this idea, Ron and coworkers have recently found that ER stress can lead to the acute production of reactive oxygen species (Harding et al., 2003). Stressing the ER in worms lacking the transmembrane kinase PERK leads to a significant accumulation of peroxides in the cell, and ablating Ero1p function drastically reduces this accumulation (Harding et al., 2003).

These observations indicate that Ero1p could be responsible for a significant proportion of reactive oxygen species (ROS) in the cell. If ~1/3 of all proteins are secretory proteins, it is reasonable to assume that one disulfide is synthesized for every

~500 amino acids translated. If the equivalent of ~3 ATP are consumed per amino acid translated, then 1 disulfide is formed for every ~1500 ATP. If the majority of ROS in the cell results from cellular respiration at 1-2% frequency, and ~4-5 ATP are produced per molecule of oxygen reduced, then 1-2 molecules of ROS are expected per 500 ATP produced through respiration. Thus, if one molecule of ROS is produced per disulfide formed, Ero1p-mediated oxidation could account for up to ~25% of cellular ROS. ROS production by Ero1p could only be detected at a frequency of ~5%, which may be a lower bound and a limitation of the detection method (Tu and Weissman, 2002). Ero1p activity would then account for a minimum ~1-2% of cellular ROS. This is likely to be an underestimate as many proteins contain large numbers of disulfides and certain cell types have enormous secretory loads. In addition, Ero1p activity is the main source of oxidized glutathione (GSSG) in the cell (Cuozzo and Kaiser, 1999; Tu et al., 2000), which contributes an additional source of oxidative stress.

Thus, Ero1p activity may be a substantial cause of oxidative stress in eukaryotic cells, necessitating proper regulation of oxidative folding and the function of reductant systems in the ER. It is important for a cell to tie protein oxidation to its folding load, since without proper regulation of oxidative folding, the ER would become overoxidized, leading to protein misfolding, the production of reactive oxygen species and oxidized glutathione, and the futile consumption of energy in the form of reducing equivalents. As the Ero1p oxidation system is highly responsive to levels of free FAD in the cell, controlling the levels of free FAD available to Ero1p may be a posttranslational mechanism to regulate oxidative folding according to the cell's needs. Interestingly, *RIB1,* which controls the first step of riboflavin biosynthesis, is a target of the UPR

(Travers et al., 2000). Preliminary experiments indicate that free FAD levels in yeast can vary according to its growth phase and conditions (Tu, B.P., unpublished data). Alternatively, free FAD levels in the ER could be controlled by a FAD-specific transporter.

In summary, while previous studies of oxidative folding have focused on the bulk redox potential of the ER, it is now evident that eukaryotic disulfide formation proceeds by the kinetic shuttling of oxidizing equivalents to folding substrates. Since the Ero1p-driven oxidation machinery is insulated from the bulk redox environment, reduced glutathione and perhaps certain PDI homologs can assist in the isomerization and reduction of incorrect disulfide bonds. Oxidative folding is coupled to the strong reduction potential of molecular oxygen through a FAD-dependent mechanism, but a potential consequence is the production of toxic reactive oxygen species. Indeed, it appears that Ero1p can be a significant contributor to cellular oxidative stress, and suggests that its activity must be carefully adjusted according to the folding load on the ER. Controlling the levels of free FAD available to Ero1p may be a means to regulate oxidative folding according to the cell's nutritional or metabolic state. Future work will reveal how the ER maintains its optimal environment for the multitude of redox processes required for the proper folding of secretory proteins.

CHAPTER 2

Biochemical Basis of Oxidative Protein Folding in the Endoplasmic Reticulum

Biochemical Basis of Oxidative Protein Folding in the Endoplasmic Reticulum

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Abstract

The endoplasmic reticulum (ER) supports disulfide bond formation by a poorly understood mechanism requiring protein disulfide isomerase (PDI) and Ero1p. In yeast, Ero1p-mediated oxidative folding was shown to depend on cellular flavin adenine dinucleotide (FAD) levels but not on ubiquinone or heme, and Ero1p was shown to be an FAD-binding protein. We reconstituted efficient oxidative folding in vitro using FAD, PDI and Ero1p. Disulfide formation proceeded by direct delivery of oxidizing equivalents from Ero1p to folding substrates via PDI. This kinetic shuttling of oxidizing equivalents could allow the ER to support rapid disulfide formation while maintaining the ability to reduce and rearrange incorrect disulfide bonds.

Proteins that traverse the secretory pathway are typically stabilized by one or more disulfide bonds. To support efficient disulfide formation, cells actively promote oxidation in the two compartments where disulfide-linked folding commonly occurs: the eukaryotic ER (Frand et al., 2000) and the bacterial periplasm (Rietsch and Beckwith, 1998). In bacteria, an electron transport pathway links disulfide bond formation to the respiratory chain (Kobayashi et al., 1997; Bader et al., 1999). The integral membrane protein DsbB oxidizes the CxxC active site of the PDI homolog DsbA, which then catalyzes disulfide formation in folding proteins. DsbB is reoxidized by ubiquinone produced during respiration.

Over the past few decades, a number of factors have been suggested to contribute to disulfide formation in the ER, including secretion of reduced thiols, uptake of oxidized thiols, and a variety of redox enzymes and small molecule oxidants (Ziegler and Poulsen, 1977; Hwang et al., 1992; Frand et al., 2000). The physiological importance of any of these to disulfide formation has not been established. Genetic studies in *Saccharomyces cerevisiae* have identified an essential and conserved ER-resident protein, Ero1p (Frand and Kaiser, 1998; Pollard et al., 1998), loss of which results in the accumulation of reduced PDI and the cessation of disulfide bond formation, a phenotype resembling that of loss of DsbB in bacteria (Bardwell et al., 1993). Ero1p, however, has no apparent homology to DsbB or any other redox enzymes. Thus whether oxidative folding occurs by a similar biochemical mechanism in eukaryotes and bacteria, or even if Ero1p can catalyze redox reactions, is not known.

To define the requirements for oxidative folding in the ER, we used reverse genetics in *S. cerevisiae* to eliminate components of the cellular redox machinery and examined the effects on disulfide-linked folding. We monitored disulfide formation by pulsing cells with the reductant dithiothreitol (DTT) and following the rate and efficiency of folding of newly synthesized carboxypeptidase Y (CPY), which contains five disulfide bonds required for folding and ER export (Stevens et al., 1982). Deletion of *COQ5* or *HEM1*, which blocks biosynthesis of ubiquinone (Barkovich et al., 1997; Poon et al., 1997) or heme (Astin and Haslam, 1977), respectively, inhibited respiration and ER-associated cytochromes, but did not alter the kinetics of CPY folding (Figure 1A). Depletion of Nfs1p, an essential protein required for iron-sulfur (Fe-S) cluster assembly (Kispal et al., 1999), also had little effect on CPY folding (Figure 1A, supplementary data). Moreover, under depletion conditions where the activities of known Fe-S cluster proteins are abolished (Kispal et al., 1999), the kinetics of Ero1p reoxidation were comparable to those of a wild type strain (Figure 1B). Finally, Ero1p was functional in yeast grown under strictly anaerobic conditions (supplementary data). Thus Fe-S clusters, molecular oxygen, ubiquinone, and hemes are not required for Ero1p-mediated oxidative folding.

We then investigated the role of riboflavin and its metabolic derivatives (flavin mononucleotide [FMN] and FAD) in oxidative folding using a strain lacking the *RIB5* gene, which is required for riboflavin biosynthesis. Depletion of riboflavin from the growth media inhibited CPY folding in a Δ*rib5* strain (Figure 1A) and caused PDI (Ho-Schleyer, S.C., unpublished data) and Ero1p to accumulate in a reduced form even in the absence of DTT (Figure 1B). Depletion of riboflavin also results in loss of FMN and FAD, which are derived from the sequential activities of Fmn1p and Fad1p, respectively

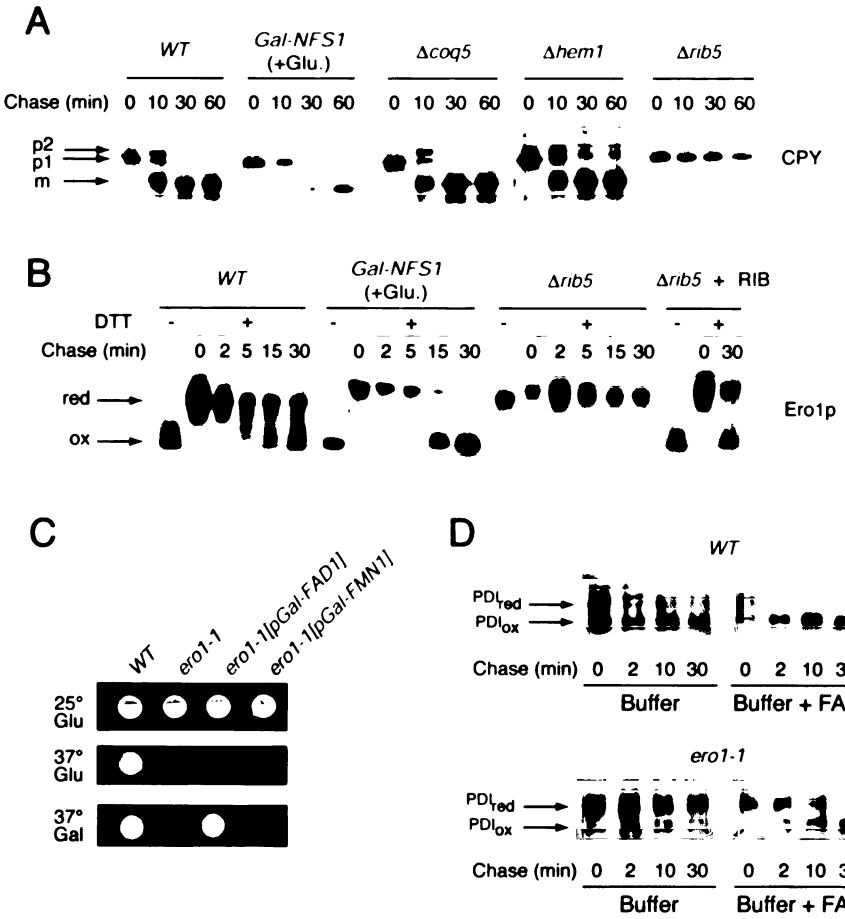

Figure 1. Oxidative protein folding is highly sensitive to cellular FAD levels

(A) Contribution of various cellular redox cofactors to oxidative folding of CPY. The indicated yeast strains were ^{35}S-methionine labeled for 7 minutes in the presence of 5 mM DTT, and then chased in the absence of DTT for the indicated times. CPY was immunoprecipitated from cell lysates and analyzed by SDS-PAGE and autoradiography (Horazdovsky and Emr, 1993). The ER form (p1) was chased to the Golgi (p2) and vacuolar mature (m) forms after DTT removal except in the Δ*rib5* strain. The Δ*coq5*Δ*hem1* double mutant also did not show a defect in CPY maturation (Ho-Schleyer, S.C., unpublished data). For a complete description of yeast strains and plasmids in this study, see supplementary data.

(B) Contribution of various cellular redox cofactors to Ero1p reoxidation. The indicated yeast strains expressing an HA epitope-tagged version of Ero1p (Pollard et al., 1998) were treated with 5 mM DTT for 30 minutes. After the indicated chase times in the absence of DTT, cells were lysed in the presence of 20 mM AMS (Frand and Kaiser, 1999) and subjected to non-reducing SDS-PAGE and Western blot analysis using an α-HA antibody. The positions of oxidized and reduced Ero1p are indicated.

(C) Overexpression of *FAD1* suppresses the temperature-sensitivity of *ero1-1*. An *ero1-1* strain carrying a vector with no insert, or an insert encoding *FMN1* or *FAD1* regulated by the *GAL1* promoter (pGal-*FMN1* or pGal-*FAD1*, respectively) (supplementary data), or an isogenic *ERO1* (WT) strain carrying an empty vector were spotted on media containing glucose at 25°C, glucose at 37°C, or galactose at 37°C.

(D) FAD induces reoxidation of PDI in microsomes. Microsomes from wild type (WT) and *ero1-1* yeast were treated with DTT, and incubated in buffer lacking DTT with or without 200 μM FAD for the indicated times. Samples were then subjected to AMS modification, SDS-PAGE, and Western-blot analysis using an α-PDI antibody. Addition of GSSG also induced reoxidation of PDI but in an Ero1p-independent manner (supplementary data).

(Wu et al., 1995). To determine whether these components are important for Ero1p-mediated folding, we examined the effect of overexpression of *FMN1* or *FAD1* on a strain containing a temperature-sensitive allele of *ERO1* (*ero1-1*) (Frand and Kaiser, 1998). Overexpression of *FMN1* led to a modest enhancement of *ero1-1* viability, whereas overexpression of *FAD1* strongly suppressed the *ero1-1* temperature-sensitive phenotype (Figure 1C). Moreover, addition of FAD to microsomes derived from wild type but not *ero1-1* yeast greatly accelerated both the rate and yield of PDI reoxidation following DTT treatment (Figure 1D), strongly arguing for a direct role of FAD in disulfide bond formation. Taken together, our results indicated that Ero1p-mediated oxidative folding is exquisitely sensitive to cellular FAD levels.

To directly assess the biochemical mechanism of oxidative folding, we developed an affinity-based purification which yielded highly purified polyoma epitope-tagged Ero1p (Ero1p-Py_2) from yeast microsomes (Figure 2A, supplementary data). Further purification of Ero1p-Py_2 using α-polyoma resin did not alter its activities in subsequent analyses, arguing that our Ero1p preparations contained no functional contaminants (Tu, B.P., unpublished data). Purified Ero1p displayed a distinct absorbance peak at 450 nm (Tu, B.P., unpublished data). Reverse-phase HPLC analysis of denatured Ero1p revealed a single fluorescence peak that co-eluted with an FAD standard (Figure 2B). Thus Ero1p itself is a flavoprotein that contains non-covalently bound FAD.

We next asked whether Ero1p could act as an oxidase in vitro by monitoring its activity on a well-characterized folding substrate, ribonuclease A (RNase A), which contains four disulfides necessary for its folding and activity (Lyles and Gilbert, 1991). In

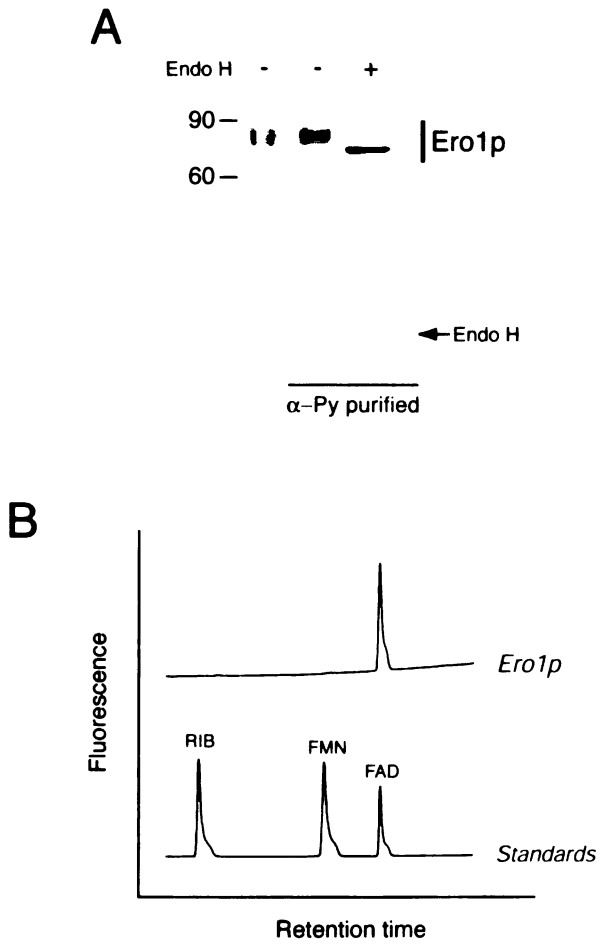

Figure 2. Ero1p is a flavoprotein

(A) SDS-PAGE analysis of purified Ero1p-Py_2 either before or after further purification on an α-polyoma column, with or without Endoglycosidase H treatment, as indicated (supplementary data). No significant differences in oxidase activity or flavin binding properties between the α-polyoma and non-α-polyoma purified protein were observed (Tu, B.P., unpublished data).

(B) Purified Ero1p contains non-covalently bound FAD. Purified Ero1p from (A) was denatured and analyzed by reverse-phase HPLC coupled to a scanning fluorescence detector (supplementary data). The stoichiometry of bound oxidized FAD to Ero1p varied between 1:4 and 1:2. A fraction of Ero1p may have been misfolded or incapable of binding FAD. For comparison, a chromatogram of standards containing riboflavin, FMN, and FAD is shown (bottom trace).

the presence of supplemental FAD and PDI, catalytic amounts of Ero1p rapidly promoted reactivation of reduced RNase A (Figure 3A). SDS-PAGE analysis directly demonstrated that the reactivation of RNase A resulted from Ero1p-mediated reoxidation of its four disulfide bonds (Figure 3B). The observed refolding of RNase A was completely dependent on Ero1p, PDI, and supplemental FAD, but did not require reduced (GSH) or oxidized (GSSG) glutathione, and was not affected by pyridine nucleotide cofactors (e.g., NAD^+, NADPH) (supplementary data). There was also a strong preference for FAD over FMN (supplementary data), in agreement with in vivo observations (Figure 1C). Increasing concentrations of Ero1p enhanced the rate of RNase A oxidative refolding (Figure 3A). Even at a stoichiometry of one Ero1p molecule per 340 RNase A disulfide bonds, refolding proceeded at a rate that was significantly faster than the refolding of RNase A in the presence of PDI and an optimal glutathione redox buffer (Figure 3A). Furthermore, an Ero1p mutant that is non-functional in vivo (Cabbibo et al., 2000; Tu, B.P., unpublished data) could not catalyze reoxidation of RNase A (Figure 3A, 3B). Thus Ero1p is an efficient oxidase that catalyzes de novo disulfide bond formation via an FAD-dependent mechanism.

In the absence of PDI, folding substrates remained reduced in vitro (Figure 3A, 3B) and in vivo (Frand and Kaiser, 1999) even with Ero1p present, suggesting that PDI acts as an intermediary in the disulfide formation process by transferring oxidizing equivalents derived from Ero1p to folding substrates. We examined whether a mutant PDI in which the second cysteine of both active sites is changed to alanine [PDI $(CxxA)_2$] could support Ero1p-mediated oxidative refolding of RNase A. This mutant PDI retains disulfide isomerase activity but cannot function as an oxidase (Laboissiere et al., 1995).

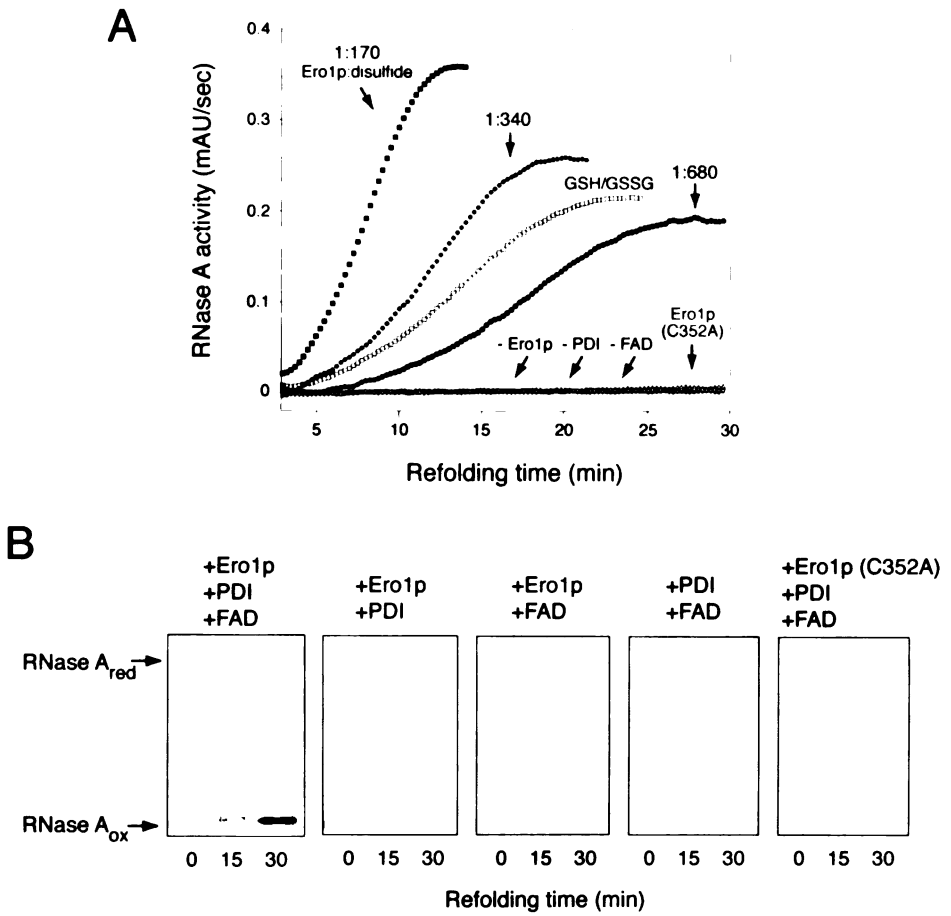

Figure 3. Ero1p is an FAD-dependent oxidase

(A) Reconstitution of oxidative protein folding in vitro. The conversion of reduced RNase A (15 μM) to its oxidized, active form in the presence or absence of the indicated concentrations of Ero1p, PDI (0.9 μM), and/or FAD (100 μM) was assayed by following hydrolysis of cCMP at 296 nm (Lyles and Gilbert, 1991). For comparison, the half-time of RNase A refolding in the presence of PDI and an optimal glutathione redox buffer (1 mM GSH, 0.2 mM GSSG) under the same conditions is ≈ 14 min. Ero1p (C352A) refers to a mutant Ero1p where Cys352 is changed to Ala.

(B) Direct observation of Ero1p-mediated catalysis of disulfide formation in RNase A. Refolding of reduced RNase A (15 μM) was followed in the absence or presence of Ero1p (0.36 μM), PDI (0.9 μM), and/or FAD (100 μM) as indicated, quenched at the indicated times with AMS, and analyzed by non-reducing SDS-PAGE.

Consistent with PDI acting as an oxidant, PDI $(CxxA)_2$ did not support RNase A refolding (Figure 4A). Surprisingly, PDI $(CxxA)_2$ was a dominant inhibitor of Ero1p-dependent oxidative folding, as inclusion of equimolar amounts of PDI $(CxxA)_2$ with wild type PDI resulted in a severe reduction of RNase A reactivation (Figure 4A). SDS-PAGE analysis revealed a disulfide crosslink between PDI $(CxxA)_2$ and Ero1p (Figure 4B), suggesting that inhibition of refolding by PDI $(CxxA)_2$ resulted from sequestration of Ero1p via a disulfide crosslink between the two proteins. A similar crosslink is observed in vivo when both Ero1p and a mutant PDI are overexpressed, albeit at much lower efficiency (Frand and Kaiser, 1999). Thus, a disulfide crosslink between PDI and Ero1p is likely to be an obligatory intermediate during the oxidation of the PDI active sites by Ero1p.

What is the role of glutathione in oxidative protein folding in the ER? Cellular GSSG production increases with Ero1p activity (Cuozzo and Kaiser, 1999). However, we found that Ero1p had no detectable activity as a direct oxidase of GSH to GSSG (Tu, B.P., unpublished data). Moreover, Ero1p-dependent folding of RNase A did not require glutathione. These considerations suggest that Ero1p-mediated oxidation of folding substrates proceeds by a protein-based relay that is largely independent of the bulk glutathione redox buffer (supplementary data). Consistent with this idea, Ero1p could efficiently drive oxidation of RNase A in the presence of a large excess of reducing agent (1 or 2 mM GSH, Figure 4C). During refolding, we then observed a gradual production of GSSG, suggesting that glutathione is not oxidized directly by Ero1p, but rather by reduction of Ero1p-derived disulfide bonds in PDI and/or substrates. Nonetheless, the glutathione buffer remained strongly reducing (e.g., at 20 min, GSH:GSSG $\approx$ 40:1)

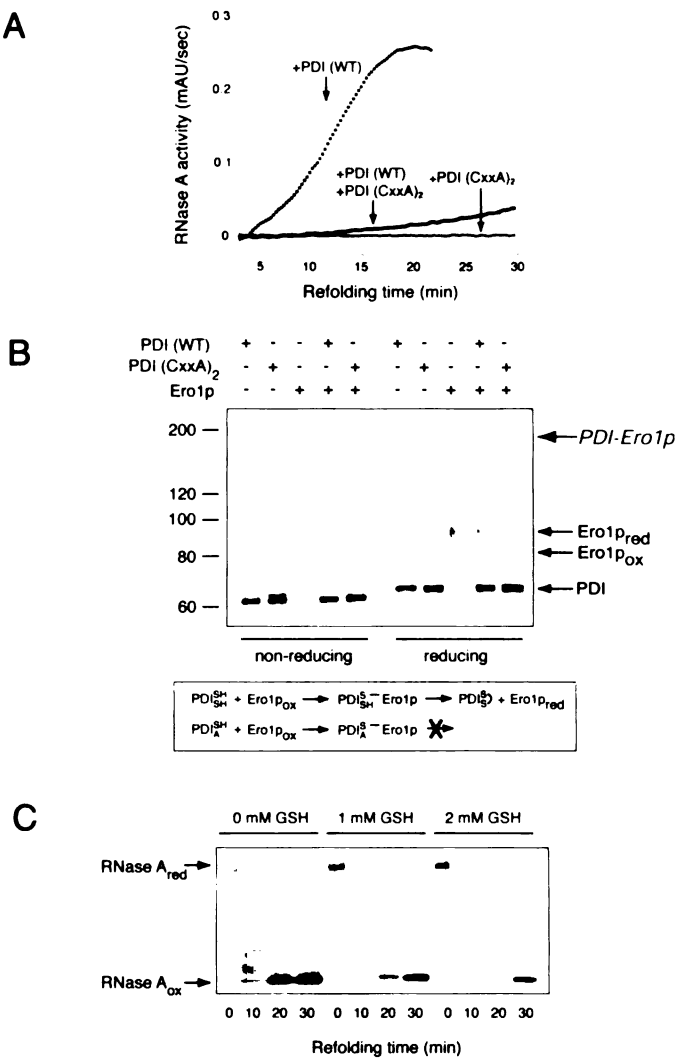

Figure 4. A protein cascade drives disulfide bond formation in the ER

(A) PDI $(CxxA)_2$ is a dominant inhibitor of Ero1p-dependent oxidative folding in vitro. Kinetics of oxidative refolding of RNase A (15 μM) in the presence of Ero1p (0.18 μM), FAD (100 μM), and WT PDI (0.9 μM), PDI $(CxxA)_2$ (0.9 μM) , or both.

(B) PDI $(CxxA)_2$ forms a disulfide crosslink with Ero1p. Following a brief incubation of Ero1p (1.3 μM) with either PDI $(CxxA)_2$ (1.7 μM) or WT PDI (1.7 μM), free cysteines were blocked and potential disulfides trapped with the addition of N-ethylmaleimide. Note the PDI-Ero1p disulfide crosslink (~200 kDa) in the sample containing Ero1p and PDI $(CxxA)_2$. Western blot analysis confirmed the presence of both PDI and Ero1p in this band (Tu, B.P., unpublished data). Below, schematic model of Ero1p-catalyzed oxidation of PDI. PDI first forms a mixed disulfide with Ero1p, which is resolved by nucleophilic attack of the second cysteine in the PDI active site, yielding oxidized PDI. When the second cysteine is not present, the PDI-Ero1p crosslinked species cannot be readily resolved.

(C) Ero1p can drive oxidative folding under reducing conditions. RNase A (15 μM) refolding was initiated in the presence of Ero1p (0.36 μM), PDI (0.9 μM), FAD (100 μM) and the indicated concentrations of reduced glutathione (GSH). At the specified times, disulfide content was monitored as described. The observed GSH:GSSG ratio (Anderson, 1985) in the 2 mM GSH reactions at 10, 20, and 30 min was ≈ 120:1, 40:1 and 17:1, respectively.

throughout the course of RNase A oxidation. These conditions were comparable to the reducing environment of the cytosol (Hwang et al., 1992), where PDI at equilibrium should be largely reduced (Lundström and Holmgren, 1993). Despite this, Ero1p- and PDI-driven oxidation of RNase A proceeded rapidly (within roughly a factor of two of the rate of oxidation without GSH) and PDI remained oxidized (Tu, B.P., unpublished data).

In summary, Ero1p is an FAD-dependent oxidase of PDI responsible for sustaining disulfide-linked protein folding in the ER. The FAD dependence of Ero1p is unexpected as Ero1p contains no known flavin-binding motifs, and DsbB, which plays an analogous role to Ero1p in the bacterial periplasm, uses ubiquinone as the proximal oxidant in a flavin-independent reaction (Bader et al., 1999). Furthermore, unlike many microsome-associated flavoproteins, which face the cytosol, Ero1p is localized within the ER lumen (supplementary data). Given the sensitivity of Ero1p-mediated oxidation to FAD levels, regulation of the amount of FAD available to Ero1p could play an important role in modulating oxidative folding in the ER.

While previous studies of disulfide bond formation in the ER often focused on the bulk redox potential of the organelle, we demonstrated that oxidizing equivalents are delivered directly from Ero1p to folding substrates via PDI, thereby allowing disulfide formation to occur rapidly even in a reducing environment. Accordingly, in vivo PDI is found predominantly in the oxidized form (Frand and Kaiser, 1999), contrary to predictions that the reduced form should be significantly populated if it were in equilibrium with the bulk ER redox buffer (Lundström and Holmgren, 1993; Frand et al., 2000). In order to support efficient disulfide-linked folding, the ER must simultaneously

be able to rapidly add disulfide bonds to unfolded proteins and remove them from misfolded proteins. The shuttling of disulfide bonds through a protein relay, by largely insulating the Ero1p-driven oxidase machinery from other redox systems, could help prevent Ero1p from interfering with the reduction or rearrangement of incorrect disulfides.

Supplementary Data

Tu, B.P. et al., "Biochemical Basis of Oxidative Protein Folding in the Endoplasmic Reticulum"
http://www.sciencemag.org/cgi/content/full/290/5496/1571/DC1

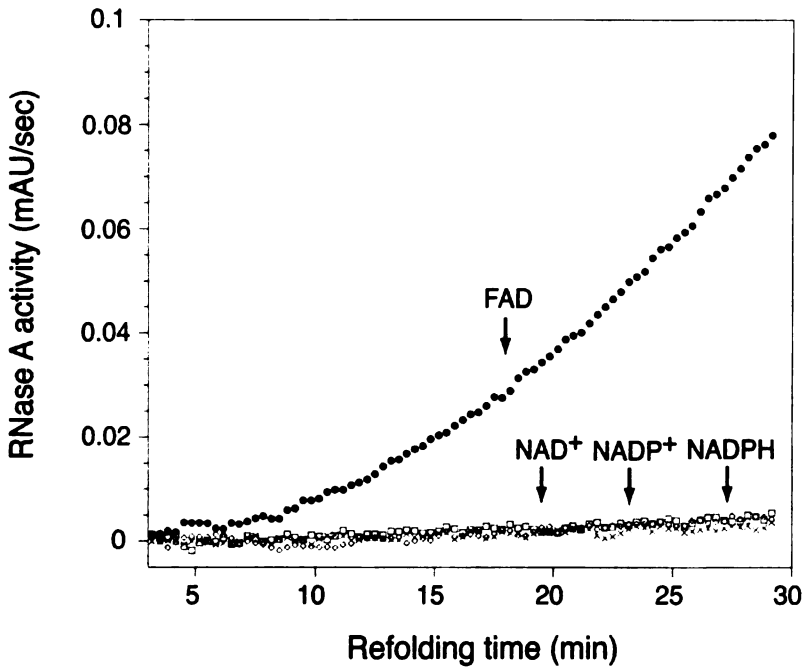

Figure 5. Ero1p specifically uses FAD as an oxidant
The oxidative refolding of reduced RNase A (12 μM) was assayed by following the hydrolysis of cCMP at 296 nm at 25°C (Lyles and Gilbert, 1991) in the presence of Ero1p (0.07 μM), PDI (0.9 μM), and either FAD (100 μM), NAD^+ (100 μM), $NADP^+$ (100 μM), or NADPH (100 μM) in a buffer containing 18 mM cCMP, 0.1 M Tris•OAc pH 8.0, 65 mM NaCl, 2 mM EDTA, and 0.005% digitonin. Note the specific requirement for FAD over these other cofactors during RNase A reoxidation catalyzed by Ero1p and PDI. These pyridine nucleotide cofactors also did not stimulate Ero1p activity in the presence of FAD (Tu, B.P., unpublished data).

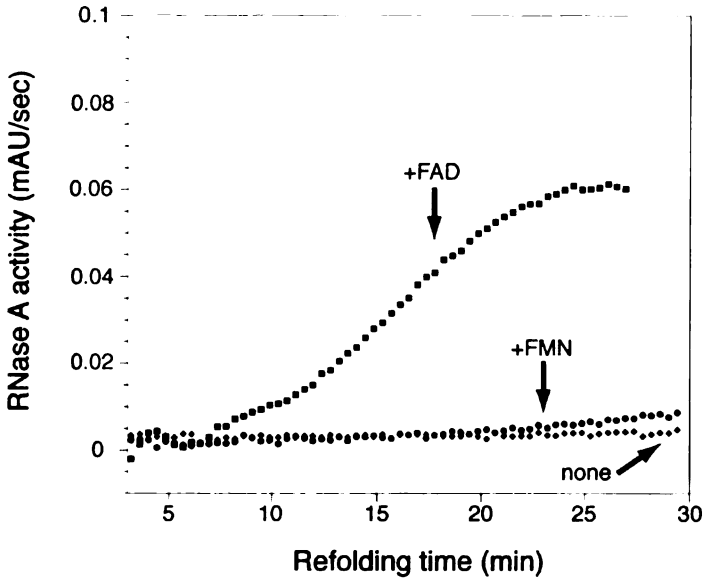

Figure 6. Ero1p shows a marked preference for FAD over FMN as an oxidant
RNase A refolding (11 μM) was assayed as in Figure 1S in the presence of Ero1p (0.11 μM), PDI (0.9 μM), and either FAD (10 μM), FMN (10 μM), or no supplemented flavins in a buffer containing 4.5 mM cCMP, 0.1 M Tris•OAc pH 8.0, 65 mM NaCl, 2 mM EDTA, and 0.005% digitonin. Note the preference for FAD over FMN during RNase A reoxidation catalyzed by Ero1p and PDI.

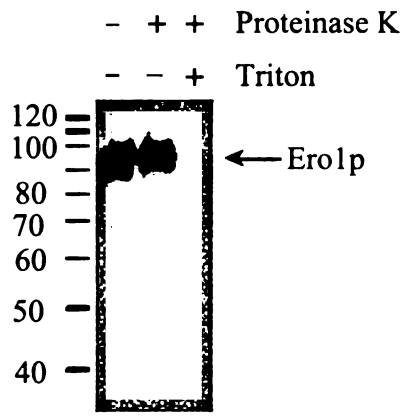

Figure 7. Ero1p is localized within the ER lumen
Microsomes were prepared as described (Brodsky et al., 1993) from strain W303-1B carrying plasmid pKT014. The microsomes were treated with or without Proteinase K at 10 mg/mL, and in the absence or presence of 1% Triton X-100, as indicated. Ero1p was visualized by Western blotting. The complete protection against digestion provided by membranes suggests that Ero1p is entirely lumenal, with little or no protein extending beyond the membrane. This observation is consistent with the fact that Ero1p contains no apparent hydrophobic transmembrane domains.

Furthermore, Ero1p purified from yeast microsomes that were similarly treated with Proteinase K in the absence of detergent remained active and still retained bound FAD, suggesting the existence of a mechanism for transporting FAD into the ER lumen (see also Figure 1D). Purified Ero1p exhibited flavin fluorescence only upon denaturation in guanidinium hydrochloride; this property is characteristic of many flavoproteins.

As has been observed in mammalian systems (Marquardt et al., 1993), GSSG supports reoxidation of folding proteins within microsomes. However, performing a similar experiment in microsomes deficient in functional Ero1p reveals that the observed reoxidation of PDI is independent of Ero1p activity. In vivo, reoxidation of PDI and folding proteins is both Ero1p-dependent and glutathione-independent (Cuozzo and Kaiser, 1999), making the physiological significance of oxidation by GSSG unclear.

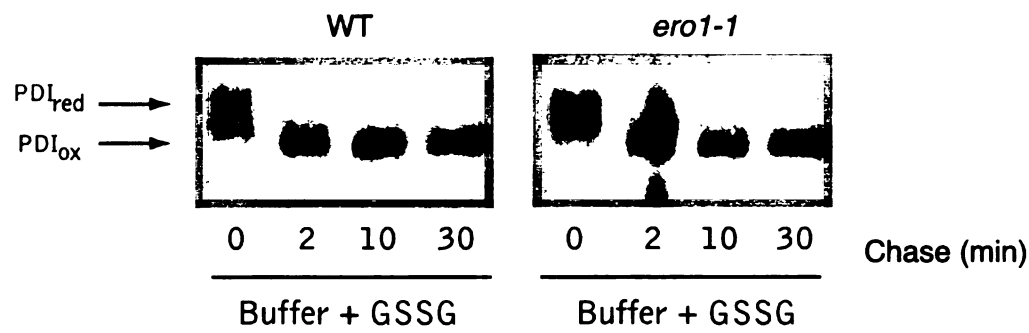

Figure 8. Addition of GSSG stimulates reoxidation of PDI independently of Ero1p activity

Microsomes were isolated from strains W303-1B and YJW594 (*ero1-1*) as described (Brodsky et al., 1993). Microsomes were treated with 20 mM DTT for 1 hour. Following removal of DTT, GSSG was added at a final concentration of 2 mM. At the indicated time points, aliquots were removed and quenched with 10% TCA. Pellets were resuspended in SDS buffer containing 20 mM AMS, treated with Endo H, and analyzed by SDS-PAGE. PDI was detected by Western analysis using an α-PDI antibody.

GSSG, which can be produced as a consequence of Ero1p activity (e.g., through GSH-mediated reduction of oxidized PDI or folding proteins), could contribute to oxidation of proteins in the ER. However, PDI, one of the most abundant proteins in the ER, is present at concentrations comparable to that of GSSG (Gilbert, 1990), and is a far better oxidant of proteins than is GSSG (Weissman and Kim, 1993). Here we also demonstrated that oxidized PDI, at a 50-fold lower concentration than GSSG, oxidized RNase A at least 8-fold faster than GSSG. Given these considerations and that Ero1p-catalyzed oxidation in vivo (Cuozzo and Kaiser, 1999) and in vitro (see main text) can occur independent of glutathione, the predominant pathway for flow of oxidizing equivalents in the ER is likely to be from Ero1p to PDI and then directly to substrate proteins (see also Figure 8).

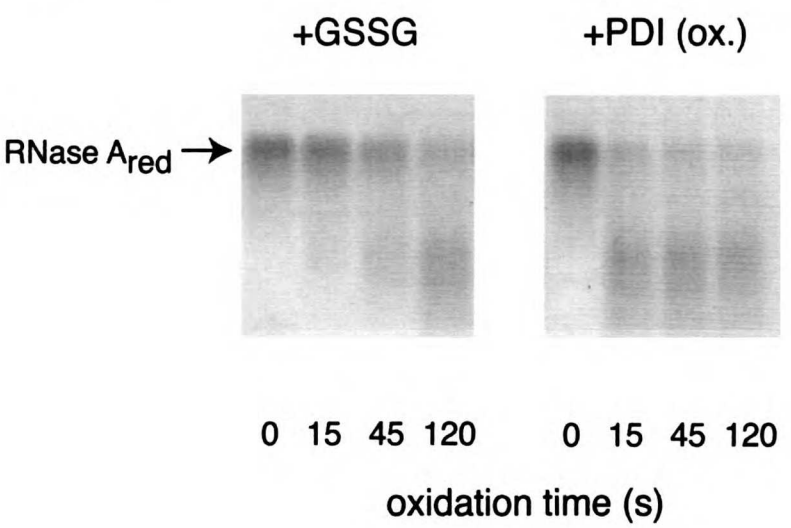

Figure 9. PDI is likely to be the predominant oxidant of proteins in the ER
Reduced RNase A (10 μM) was incubated with either GSSG (500 μM) or oxidized PDI (10 μM) in a buffer containing 50 mM Tris•Cl pH 7.3, 50 mM NaCl, 2 mM EDTA, quenched with 10 mM AMS in 1X SDS loading buffer at the indicated time points, and then analyzed using non-reducing SDS-PAGE. Oxidized PDI was prepared by incubation in the presence of 100 μM GSSG at 4°C for 30 min, followed by buffer exchange using a centrisep spin column (Princeton Separations). Note the rapid loss of the reduced form of RNase A within 15 seconds upon incubation with oxidized PDI. The PDI reaction stops short of complete RNase A oxidation because of depletion of oxidized PDI. A similar level of RNase A oxidation was achieved using a 50-fold higher concentration of GSSG as an oxidant, but after 120 seconds.

Ero1p function in the absence of molecular oxygen

Several lines of evidence indicate that Ero1p oxidase activity is not strictly dependent on molecular oxygen. First, PDI, whose oxidation in vivo is dependent on Ero1p function, persisted in a predominantly oxidized state even when yeast were grown in anaerobic conditions (Figure 10). Second, in both the presence (Pollard et al., 1998) and absence of oxygen, *ero1-2* mutant yeast were highly sensitive to DTT (Figure 11) and the *ero1-1* strain remained temperature-sensitive (Tu, B.P., unpublished data). This suggested that Ero1p function was required for viability even in the absence of oxygen. Finally, preliminary experiments in vitro indicated that purified Ero1p could catalyze the oxidation of RNase A in an FAD-dependent reaction in the absence of oxygen (Tu, B.P., unpublished data).

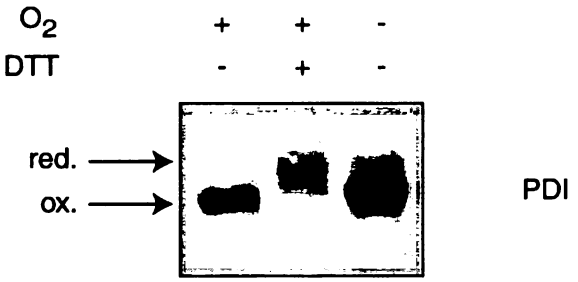

Figure 10. PDI is oxidized in yeast cells grown anaerobically
Synthetic complete media supplemented with 20 mg/L ergosterol and 1% (v/v) Tween-80 (Gollub et al., 1977) was stirred overnight in a controlled atmosphere chamber (Plas Labs) filled with an anaerobic gas mixture (85% N_2, 5% CO_2, 10% H_2). The media was determined to be oxygen-free by measurement with a dissolved oxygen sensor (Corning). YJW193 was inoculated into the media and grown anaerobically to $OD_{600} \approx 1$. Cells were treated with TCA to a final concentration of 10% (w/v) and incubated on ice for 20 min in the controlled atmosphere chamber. The samples were then removed from the chamber, spun at 14,000 rpm and washed twice with acetone. Pellets were lysed in the presence of SDS and AMS as previously described (Frand and Kaiser, 1999). As a control, YJW193 grown aerobically was treated in a similar fashion, except an aliquot of cells was exposed to 10 mM DTT for 30 min prior to exposure to TCA. The AMS-treated lysates were subjected to SDS-PAGE and Western blot analysis using an α-PDI antibody. The reduced (red) and oxidized (ox) forms of PDI are indicated with arrowheads.

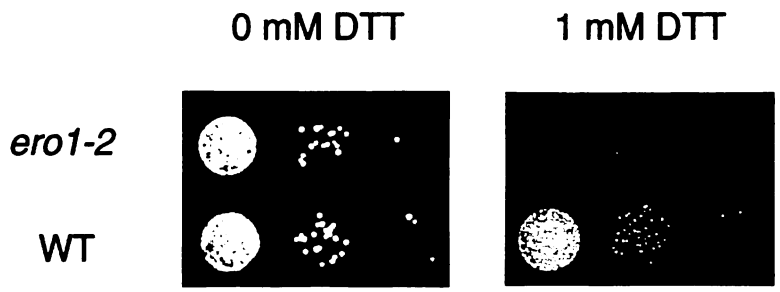

Figure 11. DTT resistance is dependent on functional Ero1p under anaerobic growth conditions
Wild type and YJW208 (*ero1-2*) strains were grown on synthetic complete plates with or without DTT under anaerobic conditions at 30°C. Each successive column represents a tenfold dilution of cells from the previous column.

Table 1. Yeast Strains

Strain	Genotype	Source/Reference
W303-1A	*leu2-3, -112; his3-11, -15; trp1-1; ura3-1; ade2-1; can1-100;* MATa	(Cox et al., 1993)
W303-1B (WT)	same as W303-1A, except MATα	(Cox et al., 1993)
Gal-NFS1	same as W303-1B, except Δ*nfs1::LEU2/Gal-NFS1*	(Kispal et al., 1999)
YJW169	same as W303-1A, except Δ*ero1::TRP1* [pKT026]	This study
YJW193	same as W303-1A, except Δ*ero1::TRP1* [pKT014]	This study
YJW208	same as W303-1A, except Δ*ero1::HIS3/ero1-2*	This study
YJW594 (*ero1-1*)	same as W303-1B, except Δ*ero1::HIS3/ero1-1*	This study
YJW598 (Δ*coq5*)	same as W303-1B, except Δ*coq5::URA3, trp1-1::TRP1 UPRE-lacZ*	This study
YJW604 (Δ*hem1*)	same as W303-1B, except Δ*hem1::LEU2, trp1-1::TRP1 UPRE-lacZ*	This study
YJW696 (Δ*rib5*)	same as W303-1B, except Δ*rib5::TRP1*	This study

All deletion strains were generated by replacing the entire open reading frame with the indicated auxotrophic marker using the Pringle method (Longtine et al., 1998). The *Gal-NFS1* strain was kindly provided by Dr. Roland Lill (Kispal et al., 1999). Unless otherwise indicated, all strains were grown in synthetic defined media (SD) which lacks ubiquinone, heme and their immediate precursors (1991). The Δ*hem1* strain was grown in the presence of 1% (v/v) Tween-80 and 20 mg/L ergosterol (Gollub et al., 1977). The growth medium for the Δ*rib5* strain contained 20 mg/L riboflavin (Santos et al., 1995).

Because *NFS1* is essential, we used a strain in which *NFS1* was controlled by a repressible promoter and assayed CPY folding after 24 hours of growth under repressing conditions. Although no Nfs1p could be detected at this time point, the kinetics of CPY folding and export from the ER were only modestly decreased compared to a wild type strain (Figure 1A). It is possible that this folding activity was due to residual Fe-S clusters. We could not examine CPY folding at later time points due to the lack of de novo protein synthesis. Instead, we followed the kinetics of reoxidation of the pre-existing pool of Ero1p following DTT treatment.

For the CPY assays shown in Figure 1A, the *Gal-NFS1* strain was grown under repressing conditions (2% glucose) for 24 hours and the Δ*rib5* strain was grown in the absence of riboflavin for 17 hours prior to labeling.

In Figure 1B, the *Gal-NFS1* strain was grown under repressing conditions for 40 hours and the Δ*rib5* strain was grown in the absence of riboflavin for 17 hours prior to analysis of the Ero1p oxidation state.

Strain and Plasmid Construction

To recover the *ero1-2* allele, plasmid pKT001 was gapped with BglII and then transformed into the YJW150 (Pollard et al., 1998). The *HIS3* gene from pRS313 was then inserted 326 bp downstream of the *ERO1* stop codon in this gap-repaired plasmid using the Seamless Cloning kit (Stratagene), producing plasmid pKT017. The *ero1-1* allele was recovered by performing inverse PCR on pKT017 using oligonucleotides that excluded exactly the coding sequence of *ERO1*, and transforming the PCR product into strain CKY559 (Frand and Kaiser, 1998). The resulting gap-repaired plasmid (pKT029) then consisted of a pRS316 backbone containing *ero1-1* with *HIS3* 326 bp past the stop codon. pKT029 was then cut with BamHI and the resulting fragment transformed into W303-1B, replacing the wild type *ERO1* locus with a cassette consisting of *ero1-1* and *HIS3*.

Plasmid pKT014, encoding an HA epitope-tagged Ero1p, was constructed by subcloning a ClaI fragment from pMP003 which contains the *ERO1* promoter and the majority of the *ERO1* coding sequence, into the ClaI sites of pMP008 (Pollard et al., 1998).

Plasmid pKT026, which contains *ERO1* tagged at its 3' end with sequences encoding a double polyoma epitope (MEYMPMEMEYMPME) (Schneider et al., 1994), a TEV protease cleavage site (ENLYFQG), and two immunoglobulin G (IgG)-binding "z" domains derived from protein A (ERO1-Py_2-zz), all under the control of the native *ERO1* promoter, was generated as follows. Complementary oligonucleotides encoding the polyoma epitope flanked by an XbaI and a NotI restriction site were annealed and

ligated at the XbaI and NotI sites of plasmid pKT014, resulting in the intermediate plasmid pKT025. The TEV protease site and ZZ-domain were generated by PCR amplification of the ZZ domain from plasmid pKSZZ (Kaffman et al., 1998) with a 5' oligonucleotide encoding the TEV protease cleavage sequence. This PCR product was ligated at the NotI and SacI sites of plasmid pKT025. This plasmid pKT026 could fully complement a Δ*ero1* strain.

Plasmid pBT005, which contains ERO1-Py_2-zz under the control of the inducible yeast *GAL1* promoter, was constructed as follows. ERO1-Py_2-zz was generated by PCR amplication from pKT026 and then subcloned into the BamHI and SacI sites of a pRS315-based backbone containing ~500 bp of upstream promoter sequence of the *GAL1* gene.

Plasmid pBT006 was constructed by subcloning the GAL1promoter-ERO1-Py_2-zz fragment from pBT005 into the XhoI and SacI sites of pRS425.

Plasmid pBT008, containing the *ERO1* gene in which cysteine 352 is changed to alanine, but otherwise identical to pBT006, was constructed using Quikchange site-directed mutagenesis (Stratagene).

Plasmid pBT101, containing a His_6-tagged derivative of yeast *PDI1* under control of the T7 promoter, was constructed as follows. *PDI1* (residues 28-522) was PCR amplified from yeast genomic DNA and subcloned into the XmaI and XhoI sites of

pBH4, a pET-19b-derived vector designed for overxpression of His_6-tagged proteins in *E. coli* (Hillier et al., 1999).

Plasmid pBT104, expressing PDI $(CxxA)_2$ where cysteine residues 64 and 409 are changed to alanine, but otherwise identical to pBT101, was constructed using site-directed mutagenesis (Stratagene).

The pGal-*FMN1* and pGal-*FAD1* plasmids were constructed by subcloning the PCR fragments corresponding to the coding region of *FMN1* or *FAD1* into the Sal1-Not1 restriction sites of the pBT005 plasmid.

NOTES

We monitored disulfide content in substrates by SDS-PAGE analysis following modification with AMS (4-acetamido-4'-maleimidylstilbene-2,2'-disulfonic acid), a thiol-modifying reagent that increases a protein's molecular weight by ~500 Da per free thiol (Kobayashi et al., 1997).

Microsomes were prepared as described and resuspended in Buffer 88 (Brodsky et al., 1993) containing 20 mM DTT for one hour, washed with Buffer 88 to remove DTT and resuspended in Buffer 88 in the presence or absence of 200 μM FAD. At the indicated times, aliquots were quenched with TCA to 10% (w/v). TCA precipitates were resuspended in 1% SDS, 50 mM Tris•Cl pH 7.5, 1 mM PMSF, containing 20 mM AMS, incubated at room temperature for 15 min, 37°C for 10 min, and boiled for 2 min prior to Endo H treatment and SDS-PAGE analysis.

Oxidative refolding was initiated by addition of reduced RNase A (Lyles and Gilbert, 1991) to the indicated concentration of purified Ero1p, bacterially expressed PDI, and/or FAD (100 μM) in a buffer containing 18 mM cCMP, 0.1 M Tris•OAc pH 8.0, 65 mM NaCl, 2 mM EDTA, and 0.005% digitonin. RNase A activity (hydrolysis of cCMP) was followed by the rate of change of absorbance at 296 nm at 25°C (Lyles and Gilbert, 1991). The disulfide content of RNase A was monitored in a similar buffer, but without cCMP. Samples were analyzed at the indicated times by the addition of SDS-PAGE buffer and 10 mM AMS, incubation for 30 min at room temperature, followed by non-reducing SDS-PAGE.

Reduced PDI (WT) or PDI $(CxxA)_2$ (1.7 μM) was added to Ero1p (1.3 μM) in a buffer containing 50 mM HEPES pH 7.5, 100 mM NaCl, 2 mM EDTA, and 0.05% digitonin. After 10 min at room temperature, free sulfhydryls were quenched by addition of SDS-PAGE buffer and 10 mM N-ethylmaleimide for 1 h. The sample was then divided into two and subjected to SDS-PAGE under reducing or non-reducing conditions

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Methods

Ero1p purification

Plasmid pBT006 or pBT008 was transformed into YJW169 for overexpression of Ero1p in yeast. This strain was grown and induced as described previously (Worland and Wang, 1989). Microsomes were then prepared from the harvested cell pellets as described (Brodsky et al., 1993). All steps hereafter were performed at 4°C unless otherwise noted. Microsomes were solubilized in 50 mM HEPES pH 7.5, 400 mM KOAc, 8 mM $Mg(OAc)_2$, 1 mM $CaCl_2$, 10% glycerol, 1 mM β-mercaptoethanol, 2% digitonin, 1 mM PMSF, 20 μM leupeptin for 1 h and then spun at 35,000 g for 12 min. The supernatant was collected and the pellet resolubilized and respun once again. The solubilized extract was then allowed to bind IgG sepharose (Pharmacia) for 1-2 h. The resin was then washed twice with 50 mM HEPES pH 7.5, 150 mM NaCl, 10% glycerol, 0.1% digitonin, 1 mM PMSF, 20 μM leupeptin, followed by a wash with the same buffer but with 300 mM NaCl. The resin was then resuspended in 50 mM HEPES pH 7.5, 100 mM NaCl, and 0.1% digitonin. TEV protease (Gibco) was then added (~0.5 U/μg Ero1p) and allowed to cleave Ero1p-Py_2 from the resin at room temperature for 1-2 h. TEV protease (which is His_6-tagged) was then depleted by incubating in the presence of Ni-NTA agarose (Qiagen) at 4°C for 30 min.

Further purification of Ero1p over α-polyoma resin

Ero1p-Py_2 from the IgG sepharose purification step was allowed to bind α–polyoma protein G sepharose in a buffer containing 50 mM HEPES pH 7.5, 100 mM NaCl, 10% glycerol, 0.1% digitonin, 1 mM PMSF for 2 h at 4°C. The α-polyoma resin

was washed twice with the same buffer, followed by a wash with the same buffer but with 300 mM NaCl. Ero1p-Py_2 was eluted off the resin by incubation in 50 mM HEPES pH 7.5, 100 mM NaCl, 0.1% digitonin, 0.002% n-octyl-β-glucoside, and 100 μg/mL polyoma peptide (EYMPME) for 10 min at room temperature.

HPLC analysis of Ero1p-bound FAD

Approximately 2-3 μg of Ero1p was denatured in 6 M guanidinium hydrochloride, 20 mM Tris•Cl pH 7.8, and analyzed using reverse-phase HPLC. Samples were run through a μRPC ST 4.6/100 C2/C18 column (Pharmacia) using a methanol/acetic acid/*t*-butylammonium phosphate solvent system (Fahey and Newton, 1987). Flavins were identified using a scanning fluorescence detector (ex. 450 nm, em. 520 nm, Waters model 474).

PDI purification from *E. coli*

Plasmid pBT101 or pBT104 was transformed into *E. coli* BL-21 for overexpression. Cells were induced with 0.4 mM IPTG at OD ≈ 0.3 for 2 h at 37°C, harvested, and resuspended in 50 mM Tris•Cl pH 7.9, 50 mM NaCl, 10% sucrose. All steps hereafter were performed at 4°C. Lysozyme (100 μg/mL) was added and the suspension incubated for 30 min on ice. DNase I (2 U) and $MgCl_2$ (5 mM) were then added. Cells were sonicated and then spun at 15,000 rpm for 20 min at 4°C. The supernatant was filtered through a 0.45 μ filter and then mixed with pre-equilibrated Ni-NTA agarose (Qiagen) for 1-2 h. The resin was washed with 10 column volumes of 50 mM Tris•Cl pH 7.9, 50 mM NaCl, 10% sucrose, 1 mM β-mercaptoethanol, 1 mM PMSF, 20 μM leupeptin, followed by 5 column volumes of 50 mM Tris•Cl pH 7.9, 300

mM NaCl, 10% glycerol, 20 mM imidazole, 1 mM β-mercaptoethanol, 1 mM PMSF, 20 μM leupeptin. The protein was eluted with the same buffer but with 100 mM imidazole. Imidazole was removed using a PD-10 gel filtration column (Pharmacia).

Footnotes

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CHAPTER 3

The FAD and O_2-Dependent Reaction Cycle of Ero1-Mediated Oxidative Protein Folding in the Endoplasmic Reticulum

The FAD and O_2-Dependent Reaction Cycle of Ero1-Mediated Oxidative Protein Folding in the Endoplasmic Reticulum

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Summary

The endoplasmic reticulum (ER) supports disulfide formation through an essential protein relay involving Ero1p and protein disulfide isomerase (PDI). We find that in addition to having a tightly associated flavin adenine dinucleotide (FAD) moiety, Ero1p is highly responsive to small changes in physiological levels of free FAD. This sensitivity underlies the dependence of oxidative protein folding on cellular FAD levels. FAD is synthesized in the cytosol but can readily enter the ER lumen and promote Ero1p-catalyzed oxidation. Ero1p then uses molecular oxygen as its preferred terminal electron acceptor. Thus Ero1p directly couples disulfide formation to the consumption of molecular oxygen, but its activity is modulated by free lumenal FAD levels, potentially linking disulfide formation to a cell's nutritional or metabolic status.

Introduction

A hallmark of secreted proteins is that they require disulfide bonds for their proper folding and function. In eukaryotes, oxidative protein folding occurs in the endoplasmic reticulum (ER) (for review, see (Fewell et al., 2001). The folding capacity of the ER can be enormous with some cell types secreting daily the equivalent of their own mass (Helenius et al., 1992). Because disulfide formation is a redox reaction, sustained disulfide-linked folding requires a continuous flux of oxidizing equivalents into the ER to match the flux of translocating proteins. While genetic approaches have identified key components of the protein machinery responsible for disulfide formation, the source of the oxidizing potential for the ER and how oxidation is coupled to folding load is not known.

Oxidative protein folding is dependent on the essential and conserved ER-resident protein Ero1p (Frand and Kaiser, 1998; Pollard et al., 1998; Cabibbo et al., 2000). Compromised Ero1p function results in sensitivity to the reductant dithiothreitol (DTT) and the accumulation of the reduced forms of protein disulfide isomerase (PDI) and folding proteins (Frand and Kaiser, 1998; Pollard et al., 1998; Frand and Kaiser, 1999). Recent studies have begun to address the mechanism by which Ero1p contributes to oxidative folding. Several common cellular redox cofactors are not required for disulfide formation including glutathione (Frand and Kaiser, 1998), which is the major small molecule thiol in the ER (Hwang et al., 1992), and ubiquinone (Tu et al., 2000), which plays a critical role in oxidative folding in bacteria (Kobayashi et al., 1997; Bader et al., 1999; Bader et al., 2000). Rather, disulfide formation is driven by a flavin-dependent reaction (Tu et al., 2000). Depletion of riboflavin, a precursor of flavin adenine

dinucleotide (FAD), rapidly leads to defects in the folding of disulfide-containing substrates (Tu et al., 2000). Ero1p itself is a FAD-binding protein that specifically oxidizes PDI; PDI then directly catalyzes disulfide formation in folding proteins (Frand and Kaiser, 1999; Tu et al., 2000). This Ero1p and PDI-driven oxidation relay supports rapid disulfide formation largely independent of the bulk redox environment (Tu et al., 2000), perhaps allowing the simultaneous reduction and rearrangement of incorrect disulfides in the ER.

In addition to Ero1p, two other ER-associated FAD-dependent oxidases have been recently suggested to contribute to oxidative folding. Erv2p was proposed to constitute a second pathway for disulfide formation based largely on its ability to suppress Ero1p defects when overexpressed (Gerber et al., 2001; Sevier et al., 2001). Erv2p belongs to the sulfhydryl oxidase family of enzymes, which use a FAD cofactor and molecular oxygen to catalyze disulfide formation in proteins directly (Hoober et al., 1999; Gerber et al., 2001; Sevier et al., 2001; Gross et al., 2002). A second oxidase, Fmo1p (YHR176W), was proposed to contribute to disulfide formation by oxidizing small molecule thiols such as glutathione (Suh et al., 1996). Fmo1p belongs to the flavin-containing monooxygenase family of enzymes, which catalyze the O_2- and NADPH-dependent oxidation of various substrates (Muller, 1991). The relative contribution of Erv2p and Fmo1p to disulfide formation in vivo remains unclear.

In the bacterial periplasm, disulfide formation is supported by DsbB and DsbA, which play analogous roles to Ero1p and PDI, respectively (Bardwell et al., 1991; Bardwell et al., 1993; Missiakas et al., 1993; for review see Ritz and Beckwith, 2001).

DsbB oxidizes DsbA, which then introduces disulfide bonds into folding proteins. DsbB is subsequently reoxidized by quinones (Bader et al., 1999; Bader et al., 2000). In a separate reaction, oxidized quinones are regenerated by the respiratory chain using O_2 or an alternate terminal electron acceptor under anaerobic conditions (Kobayashi et al., 1997; Bader et al., 1999). In this manner, quinones provide a direct link between disulfide formation in newly synthesized proteins and the cell's respiratory status. In contrast, for eukaryotic cells the extent to which disulfide formation is linked to other aspects of cellular redox chemistry is not known. Coupling of disulfide formation to load is likely to be important since unregulated oxidation could lead to protein misfolding by promoting the indiscriminate formation of disulfides, as well as other oxidative stresses.

Here we have used a combined genetic and biochemical approach to establish that Ero1p and PDI act as a "self-contained" oxidase system which directly couples disulfide formation to the consumption of molecular oxygen. We also determine the basis for the FAD-dependence of oxidative folding in the ER and explore the potential implications of this dependence for the regulation of oxidative folding.

Results

Oxidative folding in vivo is highly sensitive to levels of free FAD

We performed a synthetic lethal screen in yeast which uncovered mutations leading to lethality or poor growth in combination with the temperature sensitive *ero1-1* allele (Frand and Kaiser, 1998) (see Experimental Procedures). Several mutations in genes that likely function downstream of Ero1p gave rise to synthetic phenotypes with *ero1-1*. These genes included *PDI1*, a gene involved in the attachment of glycophosphatidyl (GPI)-anchors (*GPI16*) (Fraering et al., 2001), and a gene potentially involved in protein glycosylation (*HUT1*) (Nakanishi et al., 2001). In addition, we identified an allele of *FAD1*, the yeast adenylyltransferase that converts flavin mononucleotide (FMN) to FAD (Wu et al., 1995). The *ero1-1fad1-1* double mutant grew poorly at 30°C, whereas the *fad1-1* mutant alone grew comparably to an isogenic wild type strain (Figure 12A). Fad1p is localized in the cytosol (Wu et al., 1995); consequently, the *fad1-1* loss of function mutation likely acts upstream of Ero1p to compromise oxidative folding in the ER lumen.

Taken together with the previous observation that overexpression of *FAD1* suppresses the temperature sensitivity of *ero1-1* (Tu et al., 2000), the synthetic phenotype of *fad1-1* with *ero1-1* suggests that in vivo disulfide formation is highly sensitive to cellular FAD levels. To explore this possibility, we examined the effect of modulating Fad1p activity on cellular flavin levels, which we monitored by reverse-phase HPLC (Figure 12B). Surprisingly, we found that altering Fad1p activity had only a modest effect on overall cellular FAD levels. However, a more complete analysis revealed that the majority of total FAD (~80 percent in wild type) was tightly associated with protein and

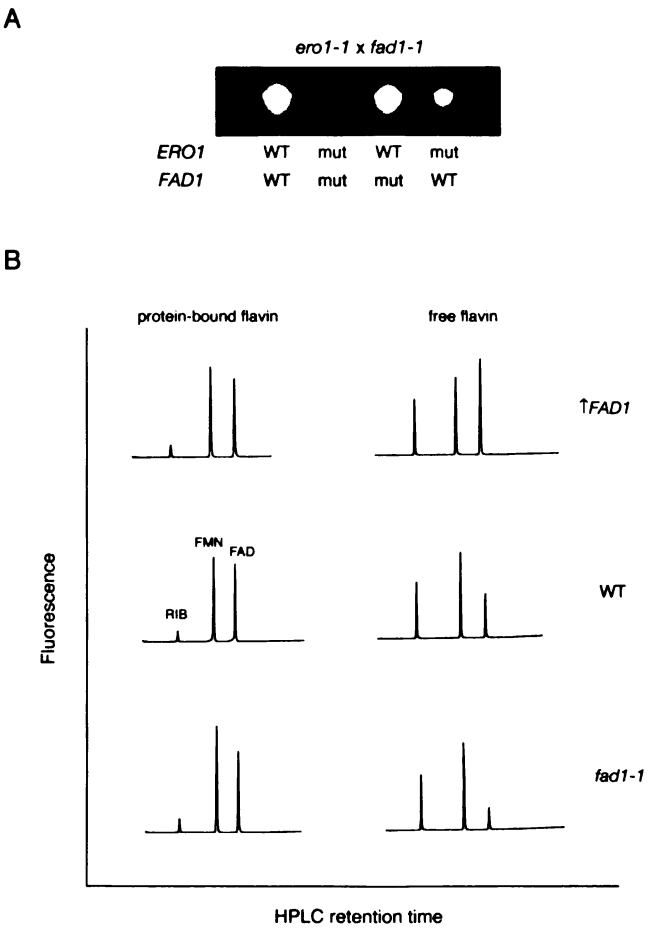

Figure 12. Oxidative folding in vivo is highly sensitive to levels of free FAD

(A) A *fad1-1* mutation causes a strong growth defect only in combination with *ero1-1*. The four spores of a tetratype tetrad derived from a cross between *ero1-1* and the *fad1-1* mutant isolated from the synthetic lethal screen were grown on rich media (YEPD) for 3 days at 30°C. The presence of the wild type (WT) or mutant (mut) allele of *ERO1* and *FAD1* for each spore is indicated below.

(B) Alteration of Fad1p activity in vivo modulates predominantly free and not protein-bound FAD levels. Wild type (WT), *fad1-1*, and wild type containing a plasmid overexpressing *FAD1* from the *ADH1* promoter (↑*FAD1*) yeast were grown in synthetic defined (SD) media to mid-late log phase, lysed and then protein-bound and free cellular flavin content analyzed using reverse-phase HPLC coupled to a scanning fluorescence detector. Note: the scale of the protein-bound and free flavin chromatograms are different. In the wild type strain, free FMN and FAD comprised ~20 percent of total FMN and FAD.

these levels were minimally affected by Fad1p activity (Figure 12B). In contrast, we observed a substantial effect on levels of free FAD, which we estimate to be ~3 μM in the wild type strain based on a total FAD concentration of ~15 μM (Gliszczynska and Koziolowa, 1998). Overexpressing Fad1p resulted in a ~2-fold increase in free FAD levels, while the *fad1-1* mutant displayed a ~2-fold decrease in free FAD levels (Figure 12B). Thus, free FAD levels severely affected the viability of yeast with a defect in the Ero1p oxidation system.

The FAD-sensitivity of oxidative folding in the ER depends on Ero1p

In principle, alteration of cellular levels of free FAD could affect oxidative folding in the ER through other flavin-dependent oxidases or a more general alteration of the metabolic or redox state of the cell. To explore these possibilites, we first established that the two other ER FAD-dependent oxidases implicated in disulfide formation, Erv2p and Fmo1p, do not have a role in the FAD-dependence of oxidative folding. Either alone or in combination, deletion of *ERV2* and *FMO1* did not result in growth defects, sensitivity to the reductant DTT, or induction of the unfolded protein response (UPR) (Tu, B.P., unpublished data). Deletion of *ERV2* and *FMO1* also did not compromise the ability of overproduction of Fad1p to suppress the temperature sensitivity of *ero1-1* (Figure 13A).

To test directly whether the effects of Fad1p overexpression require Ero1p, we took advantage of the finding that the viability of cells completely lacking *ERO1* can be rescued by deletion of *GSH1* (Sevier et al., 2001), which is involved in biosynthesis of the cellular reductant glutathione. We noted that although a *Δero1Δgsh1* haploid is viable, it grows very slowly with an estimated doubling time of ≥ 7 h on rich media at 30°C

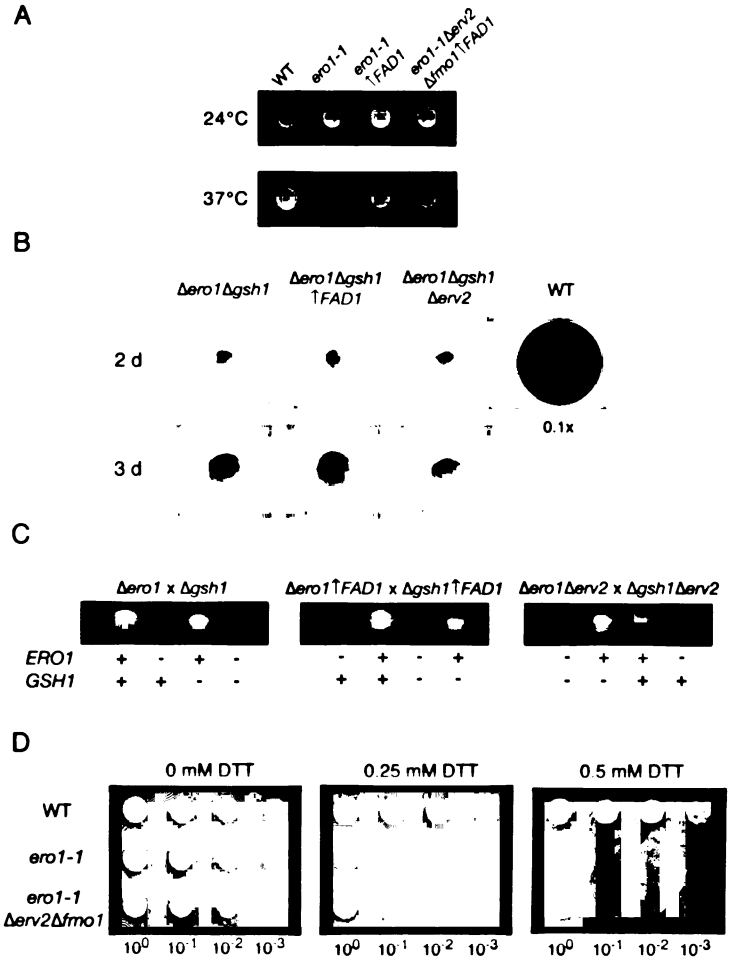

Figure 13. Ero1p drives the sole FAD-sensitive oxidative folding system in the ER

(A) Overexpression of *FAD1* suppresses the temperature sensitivity of *ero1-1* independently of the other ER FAD-dependent oxidases *ERV2* and *FMO1*. Wild type and *ero1-1* strains carrying an empty plasmid, and *ero1-1* and *ero1-1Δerv2Δfmo1* strains carrying a plasmid overexpressing *FAD1* from the *ADH1* promoter (*↑FAD1*) were grown on SD media for 2 days at the permissive (24°C) or non-permissive (37°C) temperature.

(B) Overexpression of *FAD1* does not improve growth of *Δero1Δgsh1*, and deletion of *ERV2* does not further compromise *Δero1Δgsh1*. A spore of the indicated genotype was grown on YEPD and the microcolonies that arose were photographed after 2 or 3 days at 30°C. Shown for comparison is a healthy wild type colony at 10-fold less magnification. *↑FAD1* signifies that the strain contains an integrated construct overexpressing *FAD1* from the *ADH1* promoter.

(C) A chemical chaperone can improve the growth of *Δero1Δgsh1* strains. The four spores of a tetratype tetrad resulting from the indicated cross were grown on YEPD + 10% glycerol (Figler et al., 2000) for 6 days at 30°C. The genotype of each spore is indicated below. The *Δero1Δgsh1* strains formed significantly smaller microcolonies if grown on regular YEPD media.

(D) The sensitivity of *ero1-1* to reductive stress is not increased by deletion of *ERV2* and *FMO1*. Serial 10-fold dilutions of the indicated strains were spotted onto SD media containing 0, 0.25, or 0.5 mM DTT and incubated for 2 days at 30°C.

(Figure 13B). Consistent with Δ*ero1* resulting in protein folding defects, the growth of Δ*ero1*Δ*gsh1* was improved somewhat in the presence of the chemical chaperone glycerol (Figure 13C). However, in either the presence or absence of glycerol, overexpression of Fad1p did not improve the growth of Δ*ero1*Δ*gsh1* (Figure 13B, 13C).

The ability of Δ*gsh1* yeast to grow in the absence of Ero1p also allowed us to test the proposal that Erv2p plays a "bypass" role in oxidative folding which allows yeast to survive when the Ero1p system is compromised (Sevier et al., 2001). We found that in either the presence or absence of glycerol, a Δ*ero1*Δ*gsh1*Δ*erv2* strain did not grow more slowly than the compromised Δ*ero1*Δ*gsh1* strain, suggesting that when expressed at physiological levels, Erv2p does not contribute significant levels of oxidizing equivalents to the ER even in the complete absence of Ero1p (Figure 13B, 13C). Finally, we observed that deletion of *ERV2* and *FMO1* did not increase the DTT sensitivity of *ero1-1* (Figure 13D) or affect the kinetics of disulfide formation in carboxypeptidase Y (CPY) in either the wild type or *ero1-1* background (Tu, B.P., unpublished data). Taken together, these results argue strongly that the Ero1p-driven oxidation system is the sole FAD-sensitive disulfide formation pathway in vivo.

Free FAD readily enters intact microsomes and binds specifically to Ero1p

The observation that free FAD levels determined by the cytosolic enzyme Fad1p modulate the activity of Ero1p, which is localized entirely within the ER (Frand and Kaiser, 1998; Pollard et al., 1998; Tu et al., 2000), suggests that FAD can cross the ER membrane and enter the lumen. In order to directly observe this transport, we synthesized a radioactive, photo-activated crosslinkable FAD analog, azido-FAD (FAD-2-

$N_3[\beta\text{-}^{33}P]$) (Figure 14A). Azido-FAD was comparable to FAD in its ability to support Ero1p-catalyzed refolding of reduced ribonuclease A (RNase A) (Tu, B.P., unpublished data). After incubation of azido-FAD with equivalent concentrations of purified Ero1p and bovine serum albumin (BSA) and activation of the crosslinker, a specific azido-FAD crosslink to Ero1p could be detected (Figure 14B). This crosslink could be competed by the addition of FAD but not FMN (Tu, B.P., unpublished data), confirming that Ero1p itself is a specific FAD-binding protein.

We next used azido-FAD to establish directly that FAD can enter the ER lumen and label Ero1p. Azido-FAD was added to purified microsomes for a short period (15 min), and then photoactivated to assess entry (Figure 14C). A highly specific crosslink to Ero1p could be detected in microsomes overexpressing Ero1p, indicating that azido-FAD entered the microsomes and bound to Ero1p (Figure 14C). Western analysis also confirmed that the crosslinked band corresponded to Ero1p (Figure 14C, middle). Furthermore, the azido-FAD crosslink to Ero1p was not affected by proteinase-K treatment of microsomes, but was eliminated upon treatment with proteinase-K in the presence of detergent. The addition of ATP did not noticeably affect the kinetics of entry (Tu, B.P., unpublished data). To confirm the specificity of azido-FAD crosslinks, the two lower crosslinked bands (Figure 14C) were purified using FAD-immobilized agarose and identified using mass spectrometry analysis as derivatives of cytochrome b5 reductase (Cbr1p), a known ER-associated FAD-binding protein. Thus, free FAD derived from the cytosol can readily access and equilibrate with the ER, presumably by way of protein-based pores.

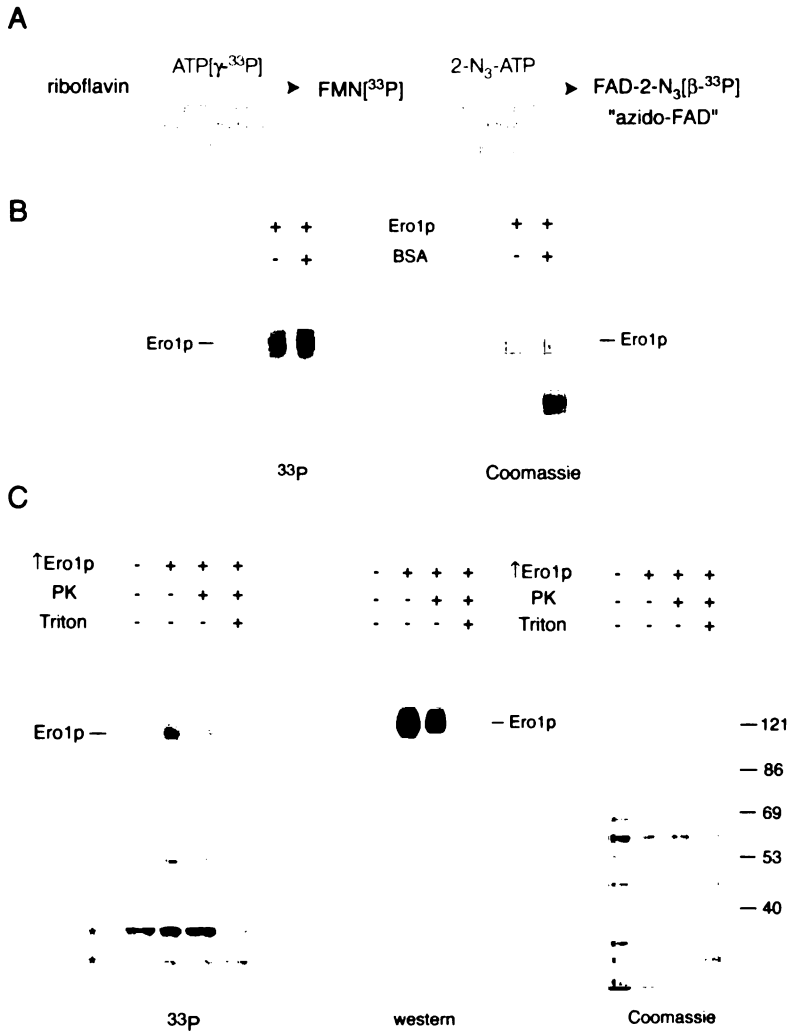

Figure 14. Free FAD readily enters intact microsomes and binds specifically to Ero1p

(A) Two-step synthesis scheme of azido-FAD (FAD-2-N_3[β-^{33}P]), a radioactive, crosslinkable FAD analog. Riboflavin was first phosphorylated to form FMN[^{33}P] using *C. ammoniagenes* FAD synthetase and limiting ATP[γ-^{33}P]. FMN[^{33}P] was then converted to azido-FAD by addition of excess 2-azido-ATP and Mg^{2+}.

(B) Azido-FAD specifically crosslinks to Ero1p. Equal concentrations of purified Ero1p and BSA were incubated with 10 μM azido-FAD for 30 min and then unbound azido-FAD removed using a spun column. The azido-FAD crosslinker was then activated by UV and crosslinked proteins were detected by SDS-PAGE and phosphorimaging (^{33}P).

(C) Azido-FAD can readily enter intact ER-derived microsomes and crosslink to Ero1p. Microsomes were prepared from wild type and Ero1p-zz-overexpressing yeast and then incubated with 10 μM azido-FAD following the indicated treatments (Proteinase K and/or 1% Triton X-100). The microsomes were washed after 15 min and then the crosslinker was activated. Total microsomal proteins were separated by SDS-PAGE and then analyzed by phosphorimaging (^{33}P), western blotting (western), and staining by Coomassie Blue (Coomassie). The asterisks (*) denote Cbr1p, a known ER-associated FAD-binding protein.

Ero1p activity in vitro is highly dependent on the concentration of free FAD

We next took advantage of the reconstituted oxidative folding reacton to investigate whether the sensitivity of Ero1p-mediated oxidative folding to levels of free FAD in vivo could be recapitulated in vitro with pure proteins. We monitored the catalysis of RNase A reoxidation (~12.5 μM = 50 μM disulfides) by purified Ero1p and PDI using concentrations of FAD ranging from 0 to 50 μM, which is more than 10-fold higher than approximate free physiological levels (Figure 15A). As found previously, no recovery of RNase A activity was observed without the addition of supplemental FAD (Tu et al., 2000). The extent of recovery of RNase A activity ranged from ~20 percent to ~90 percent in the presence of free FAD concentrations ranging from 1 to 10 μM, demonstrating that the ability of purified Ero1p to catalyze disulfide formation was extremely sensitive to levels of free FAD within this range. In particular, a two-fold increase or decrease in FAD levels from concentrations found in vivo (i.e. 3 μM) strongly affected Ero1p activity in vitro. In contrast, there was not a significant improvement of Ero1p activity upon increasing the FAD concentration from 10 μM to 50 μM. Thus, Ero1p is directly sensitive to FAD levels in a physiological range, although it remains possible that other components of the Ero1p pathway also contribute to the sensitivity to free FAD observed in vivo.

The sensitivity of Ero1p activity to free FAD levels is uncharacteristic of most flavoproteins, which typically contain tightly bound flavin cofactors (Muller, 1991). Indeed, Ero1p also contains tightly associated FAD, as up to 50 percent of Ero1p purified from yeast microsomes stably retains bound FAD (Tu et al., 2000). One explanation for the requirement for free FAD is that FAD acts as the sole oxidant and terminal electron

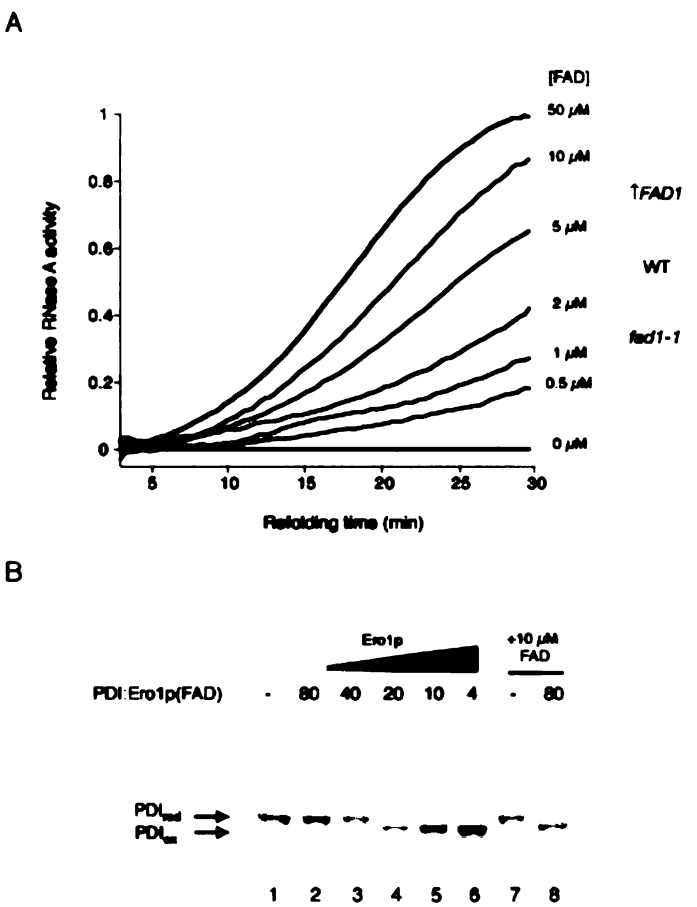

Figure 15. Ero1p activity in vitro is highly dependent on free FAD levels
(A) Ero1p-catalyzed refolding of reduced RNase A is highly sensitive to concentrations of free FAD. Refolding of RNase A catalyzed by Ero1p and PDI was monitored in the indicated concentrations of free FAD. For each condition, activity was normalized against the activity observed using 50 μM FAD, taken to be 1. The approximate in vivo concentration of free FAD in wild type, *fad1-1*, and *FAD1*-overexpressing strains is indicated on the right.
(B) FAD-bound Ero1p can support multiple rounds of PDI oxidation. Various concentrations of FAD-bound Ero1p were incubated with reduced PDI $[AxxA]_1$ for 5 min without (lanes 1-6) or with (lanes 7-8) a subsequent pulse of 10 μM free FAD. Oxidized and reduced PDI were distinguished by non-reducing SDS-PAGE following AMS modification.

acceptor for Ero1p-mediated oxidation. This would then suggest that a FAD molecule bound to Ero1p should support at most a single round of disulfide formation. To investigate this possibility, we determined how many rounds of PDI oxidation each FAD-bound Ero1p molecule could support in the absence of free FAD. We monitored the oxidation of PDI by blocking free cysteine residues with 4-acetamido-4'-maleimidylstilbene-2,2'-disulfonic acid (AMS), which retards the migration of reduced species during SDS-PAGE. To simplify the analysis of oxidized forms of PDI, we used a mutant PDI (PDI $[AxxA]_1$) in which the cysteine residues of the first CxxC active site were mutated to alanine. PDI $[AxxA]_1$was fully active in supporting RNase A oxidation by Ero1p (Tu, B.P., unpublished data).

Substoichiometric amounts of FAD-bound Ero1p were added to a constant concentration of reduced PDI $[AxxA]_1$ to initiate oxidation. At a ratio of PDI:FAD-Ero1p of ~4:1 (PDI:Ero1p ~ 2:1), PDI was oxidized within 5 min (Figure 15B). Partial oxidation of PDI (~50%) was still observed at a ratio of PDI:FAD-Ero1p of ~20:1, suggesting that each FAD-bound by Ero1p was capable of supporting at least 10 rounds of PDI oxidation. However, at the lowest stoichiometries of FAD-Ero1p to PDI (40-80:1), little PDI oxidation above background air oxidation could be detected even though FAD remained associated with Ero1p (Tu, B.P., unpublished data). Subsequent addition of 10 μM free FAD to the 80:1 reaction restored robust PDI oxidation (Figure 15B), indicating that free FAD could trigger existing FAD-bound Ero1p to sustain rapid PDI oxidation. Nonetheless, the finding that each FAD-bound Ero1p can promote oxidation of multiple PDI disulfides argues that FAD cannot be functioning as the sole oxidant

under these conditions, and suggests a role for O_2, the other oxidant present, in Ero1p-catalyzed disulfide formation.

Molecular oxygen is the terminal electron acceptor for Ero1p-catalyzed disulfide formation in vitro

To investigate the role of O_2 in Ero1p-mediated oxidative folding, we examined the ability of Ero1p and PDI to catalyze oxidation of reduced RNase A under anaerobic conditions. In the presence of 5 μM FAD under anaerobic conditions, Ero1p and PDI catalyzed the formation of only a small fraction of disulfides in reduced RNase A, with minimal RNase A oxidation occurring after 20 minutes (Figure 16A). It is possible that trace oxidants remain in the buffer or that FAD alone can support a less efficient and poorly sustained reaction. Significantly, a 20-fold increase of free FAD did not improve the rate or yield of RNase A oxidation. In contrast, with equivalent concentrations of FAD under aerobic conditions, Ero1p and PDI-catalyzed oxidation of RNase A was sustained and more robust (Figure 16A). Thus, FAD is not an efficient terminal oxidant for Ero1p, as free FAD alone cannot drive Ero1p-mediated disulfide formation. This is in marked contrast to the bacterial system in which quinones alone are sufficient to drive disulfide formation, and the role of O_2 is to regenerate oxidized quinones in a separable reaction carried out by the respiratory chain (Bader et al., 1999; Bader et al., 2000).

We next directly monitored the consumption of O_2 during Ero1p-catalyzed reoxidation of RNase A using a fluorescence-based oxygen detection system. Since the concentration of O_2 in solution at room temperature is ~240 μM, we measured O_2 levels during refolding of ~60 μM (240 μM disulfides) reduced RNase A. We observed a strong

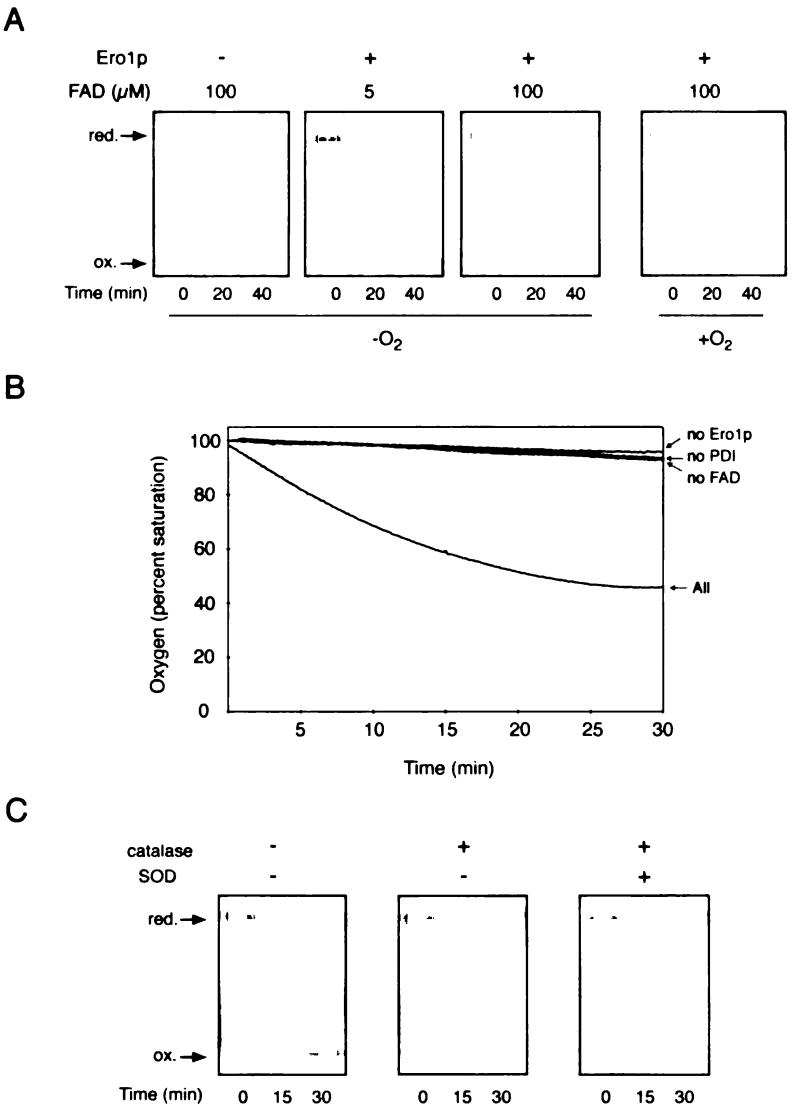

Figure 16. Molecular oxygen is a terminal electron acceptor for Ero1p-catalyzed disulfide formation in vitro

(A) Ero1p-catalyzed oxidation of RNase A is compromised under anaerobic conditions. The reoxidation of RNase A by Ero1p and PDI was initiated in the indicated concentrations of FAD under anaerobic or aerobic conditions, quenched at the indicated time points with AMS, and analyzed by non-reducing SDS-PAGE.

(B) Ero1p consumes O_2 during catalysis of RNase A oxidation. The reoxidation of reduced RNase A (60 μM) was performed in a sealed vial with the indicated components (Ero1p, PDI, and/or FAD), and O_2 levels were recorded continuously for 30 min using a fluorescence-based oxygen detecting system.

(C) Hydrogen peroxide and superoxide do not contribute substantially to Ero1p-catalyzed disulfide formation. The reoxidation of reduced RNase A by Ero1p, PDI, and FAD was performed in the presence of catalase or superoxide dismutase as indicated, quenched at the indicated time points with AMS, and analyzed by non-reducing SDS-PAGE.

time-dependent consumption of dissolved O_2 during the course of RNase A refolding; this consumption was completely dependent on all of the components of the Ero1p oxidation system (FAD, Ero1p, PDI) (Figure 16B). We confirmed by SDS-PAGE that the RNase A was efficiently oxidized during the course of this reaction (Tu, B.P., unpublished data). Roughly half of the O_2 in solution was consumed to form 240 μM disulfides, corresponding to approximately two disulfides formed per molecule of O_2. Interestingly, we could not detect significant production of hydrogen peroxide during the reoxidation reaction (~1 molecule per 20 disulfides formed, see Experimental Procedures). Moreover, hydrogen peroxide and superoxide did not contribute significantly to the observed oxidation since catalase or superoxide dismutase together with catalase had only a modest effect on Ero1p-catalyzed disulfide formation (Figure 16C). These observations suggest that the Ero1p reaction cycle is mechanistically distinct from the sulfhydryl oxidases, and rules out significant uncatalyzed air oxidation of reduced flavins. Thus, in the presence of free FAD, Ero1p can efficiently couple its reoxidation directly to the reduction of O_2.

Molecular oxygen is the preferred terminal electron acceptor for oxidative folding in vivo

The ability of purified Ero1p to use O_2 as a terminal electron acceptor in vitro prompted us to investigate the role of O_2 in oxidative folding in vivo. Ero1p is still required for viability during anaerobic growth; therefore its activity does not absolutely require O_2 (Tu et al., 2000). However, it is not uncommon for flavin-dependent oxidases to accept other oxidants, albeit with different efficiencies (Massey, 2000).

Several lines of evidence indicate that Ero1p function in vivo is compromised under anaerobic conditions. First, an *ero1-1* mutant which grows robustly at 30°C under aerobic conditions completely failed to grow at 30°C under anaerobic conditions (Figure 17A). Moreover, the anaerobic sensitivity of *ero1-1* was suppressed by overexpression of *FAD1* and was not dependent on *ERV2* or *FMO1*, arguing that the failure to grow was due specifically to compromised Ero1p function (Figure 17A). Finally, even in the presence of wild type Ero1p, decreased levels of free FAD caused by the *fad1-1* mutant led to compromised growth and sensitivity to DTT under anaerobic but not aerobic conditions (Figure 17B). The fact that Ero1p activity remained sensitive to free FAD levels under anaerobic conditions indicates that this aspect of the Ero1p reaction mechanism is independent of the terminal electron acceptor.

Interestingly, overexpression of Erv2p, which consumes O_2 in vitro (Sevier et al., 2001), was able to suppress the growth defect of *ero1-1* even under anaerobic conditions (Figure 17C), demonstrating that like Ero1p, Erv2p is capable of using an alternate terminal electron acceptor in the ER. Future identification of these terminal electron acceptors should provide insight into how disulfide formation is supported under anaerobic growth conditions.

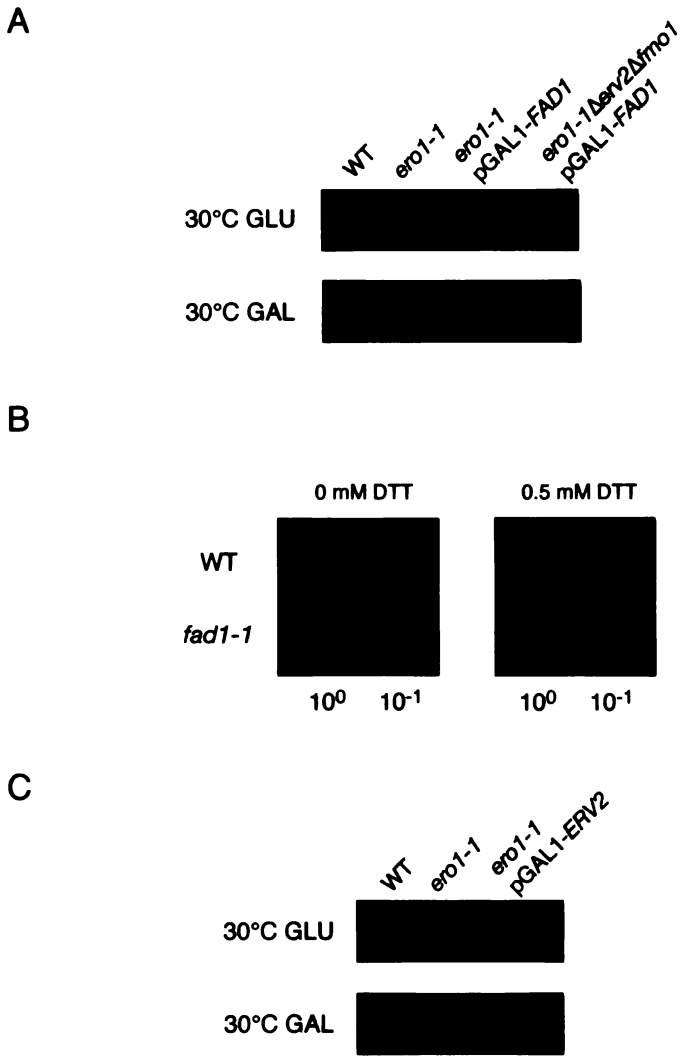

Figure 17. Molecular oxygen is the preferred terminal electron acceptor for oxidative folding in vivo

(A) An *ero1-1* strain is not viable under anaerobic conditions and overexpression of *FAD1* suppresses the anaerobic sensitivity of *ero1-1*. Wild type, *ero1-1* carrying an empty plasmid, and *ero1-1*, *ero1-1Δerv2Δfmo1* carrying a plasmid overexpressing *FAD1* from the galactose-inducible *GAL1* promoter (pGAL1-*FAD1*) were spotted on rich media containing glucose (GLU) or galactose/raffinose (GAL) and incubated for 2 days at 30°C in an anaerobic chamber.

(B) A *fad1-1* mutation exhibits a growth defect and is sensitive to DTT under anaerobic conditions. Wild type or *fad1-1* yeast were spotted on YEPD containing 0 or 0.5 mM DTT and incubated for 2 days at 30°C in an anaerobic chamber.

(C) Overexpression of *ERV2* suppresses the anaerobic sensitivity of *ero1-1*. Wild type, *ero1-1* carrying an empty plasmid, and *ero1-1* carrying a plasmid overexpressing *ERV2* from the inducible *GAL1* promoter (pGAL1-*ERV2*) were spotted on rich media containing glucose (GLU) or galactose/raffinose (GAL) and incubated for 2 days at 30°C in an anaerobic chamber. The suppression of the *ero1-1* anaerobic growth defect indicates Erv2p is capable of using an alternate electron acceptor besides O_2.

Discussion

We have determined the FAD and O_2-dependent reaction cycle of oxidative protein folding in eukaryotes. The ER supports the formation of disulfide bonds in newly translocated proteins through a protein disulfide relay involving Ero1p and PDI (Figure 18). Ero1p is a specific oxidase of PDI, which subsequently introduces disulfide bonds into folding proteins (Frand and Kaiser, 1999; Tu et al., 2000). FAD-bound Ero1p can then couple its reoxidation to the reduction of molecular oxygen. In this manner, Ero1p is able to directly harness the oxidizing potential of molecular oxygen for the formation of protein disulfide bonds in a reaction reminiscent but mechanistically distinct from other FAD-dependent oxidases with tightly associated FAD cofactors (Muller, 1991). However, a unique feature of the Ero1p oxidation system is its remarkable sensitivity to physiological levels of free FAD. High levels of free FAD stimulate the Ero1p-driven oxidative folding cycle, whereas low levels of free FAD hinder the cycle, resulting in a weakly active form of Ero1p.

We also established the existence of a robust transport system for the import of FAD into the ER lumen that allows levels of free FAD determined by the cytosolic enzyme Fad1p to modulate oxidative folding. This transport system appears to be energy-independent and facilitates the rapid equilibration of free FAD between the cytosol and ER lumen. Transport of other charged molecules such as calcium ions and ATP into the ER are thought to be mediated by specific protein channels (Clairmont et al., 1992; Guillen and Hirschberg, 1995; Stokes and Wagenknecht, 2000). A carrier protein Flx1p that transports FAD into mitochondria has been identified

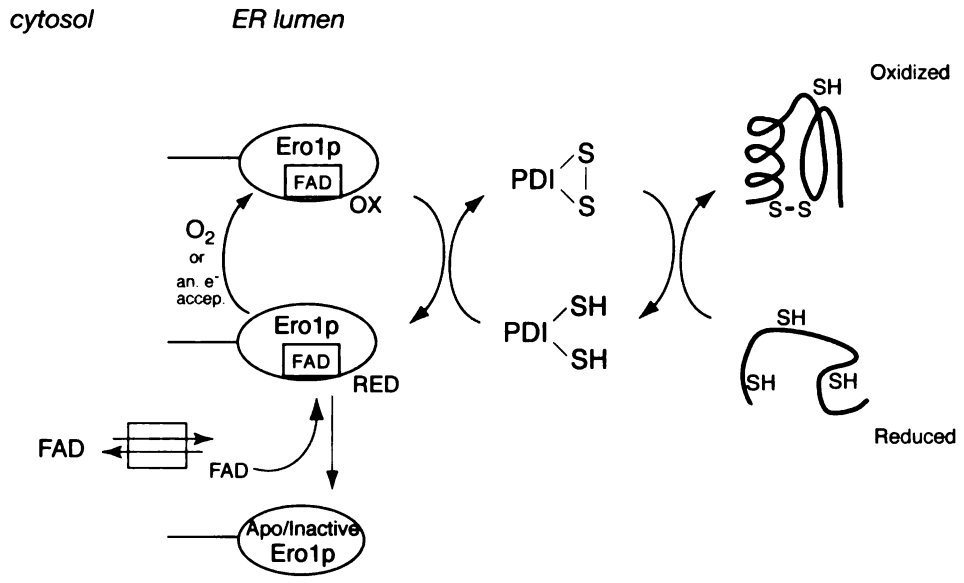

Figure 18. A model for Ero1p-mediated oxidative folding in the endoplasmic reticulum

Ero1p oxidizes PDI and then PDI oxidizes folding proteins through a sequence of disulfide exchange reactions. FAD-bound Ero1p can then directly utilize O_2 or an alternate terminal electron acceptor under anaerobic conditions to reoxidize itself. Free FAD from the cytosol can enter the ER lumen. At high free FAD levels, Ero1p is fully active, whereas at low levels Ero1p activity is compromised. "Apo" refers to a state where Ero1p may lack a weakly-associated FAD, but retains a tightly-associated FAD (see text).

(Tzagoloff et al., 1996). Identification of a FAD-specific transporter for the ER may provide important insights into the possible regulation of oxidative folding (see below).

What is the molecular basis of the sensitivity of Ero1p to free FAD levels? The following considerations are consistent with there being two or more FAD-binding sites with distinct affinities for FAD that control the transfer of electrons from Ero1p to O_2. First, Ero1p has a tight FAD binding site as even after extensive purification, Ero1p retains bound FAD. Second, FAD-bound Ero1p can support multiple rounds of PDI oxidation even in the absence of free FAD, and FAD appears to remain associated with Ero1p throughout the PDI oxidation reaction. Nonetheless, the addition of low micromolar levels of free FAD dramatically enhances catalysis of disulfide formation by FAD-bound Ero1p. This free FAD is unlikely to enhance disulfide formation by acting as the terminal electron acceptor since a stoichiometric excess cannot support oxidation under anaerobic conditions, and O_2 is normally consumed in the reaction. Hence, a weak-affinity FAD-binding site in Ero1p could be responsive to physiological levels of free FAD and function as a FAD "sensor" to modulate a separate strong affinity FAD-binding site during the course of PDI oxidation. The strong-affinity FAD-binding site could then be poised to couple the reoxidation of bound FAD to a terminal electron acceptor such as O_2. A detailed mapping of the number and location of the FAD-binding site(s) of Ero1p should lend insight into the mechanism of FAD-sensitivity.

Regardless of the precise mechanism of Ero1p's FAD sensitivity, the dependence on free FAD could provide an important means of coupling disulfide formation to the metabolic or nutritional status of the cell. It is important for a cell to tie protein oxidation

to load, since without proper regulation of oxidative folding, the ER would become overoxidized, leading to protein misfolding and the futile consumption of energy in the form of reducing equivalents. Uncontrolled Ero1p oxidase activity could also cause a more generalized oxidative stress in the cell. Consistent with this idea, Ron and coworkers have recently found that ER stress can lead to acute production of reactive oxygen species (Harding et al., 2003), perhaps through the induction of Ero1p and PDI. In bacteria, oxidative folding in the periplasm is coupled to electron transport during respiration through the oxidation of quinones, thus providing a link between the formation of protein disulfide bonds and cellular metabolism (Kobayashi et al., 1997; Bader et al., 1999; Bader et al., 2000). Since in eukaryotes oxidative folding and respiration are confined to separate organelles, the Ero1p oxidation system has evolved to function independently of respiration and adopted the use of flavin-based redox chemistry (Tu et al., 2000). In contrast to quinones, FAD is a relatively weak oxidant due to a low redox potential, but the ability of FAD-bound Ero1p to rapidly pass electrons directly to O_2 provides the driving force for disulfide formation in the ER. The efficient use of the terminal electron acceptor O_2 by FAD-bound Ero1p could explain how the oxidation of millimolar concentrations of PDI in the ER (Gilbert, 1990) is achieved despite FAD concentrations in the low micromolar range.

How then is protein oxidation in the ER controlled with the apparently self-contained Ero1p oxidation system? The unusual sensitivity of oxidative folding to levels of free FAD could allow for regulation through controlling levels of free FAD available to Ero1p. Very little is known about the cellular regulation of free FAD levels; however, several observations suggest that cellular FAD levels might change in response to

particular stimuli. The ATP-dependent conversion of FMN to FAD by *FAD1* is reversible (Efimov et al., 1998), suggesting that cellular ATP levels could affect FAD levels. As yeast stop proliferating and enter stationary phase, during which the load on ER folding drops, levels of cellular riboflavin decrease dramatically (Tu, B.P., unpublished data). Moreover, starvation of yeast for adenine causes levels of free FAD to drop rapidly upon entry into stationary phase (Tu, B.P., unpublished data). Alternatively, regulation of oxidative folding via free FAD levels could occur at the level of the ER, perhaps by manipulating the activity of a FAD-specific transporter.

Ero1p-independent pathways for disulfide formation in the ER?

It is well-established that Ero1p plays an essential role in disulfide-linked folding in eukaryotes. Recently, however, the observation that deletion of the gene responsible for biosynthesis of the reductant glutathione (*GSH1*) allowed cells lacking Ero1p to survive was interpreted to support the existence of an alternate oxidation system (Sevier et al., 2001). It has been suggested that the sulfhydryl oxidase Erv2p, which can compensate for Ero1p when expressed at super-physiological levels, may provide such an important "bypass" oxidation pathway (Sevier et al., 2001). However, we show that even in *Δero1Δgsh1* strains, loss of Erv2p does not affect growth, indicating that Erv2p is not the bypass oxidant. Moreover, several additional observations raise significant questions as to whether Erv2p plays a general role in disulfide formation. First, we observe no defect in growth, folding of CPY, or enhancement of DTT sensitivity in *Δerv2* in several strain backgrounds. In vivo disulfide formation requires PDI (LaMantia and Lennarz, 1993; Frand and Kaiser, 1999), but unlike Ero1p, Erv2p can directly oxidize disulfide bonds in folding proteins (Gerber et al., 2001). In vitro Erv2p appears to be roughly 100-fold less

active than Ero1p at oxidizing PDI (Tu et al., 2000; Sevier et al., 2001), and in contrast to Ero1p, Erv2p is not induced by the unfolded protein response (Travers et al., 2000). Finally, ER-localized ERV family members have not been found outside of yeast. Nonetheless, these observations do not exclude the possibility that Erv2p contributes to disulfide formation in a subset of proteins or under cellular conditions not examined here.

More generally, it remains an open question whether there is a specific, alternate oxidation system responsible for survival of *Δero1Δgsh1*. For example, in the highly reducing bacterial cytosol, elimination of the active reduction systems is sufficient to allow air oxidation of disulfide bonds in folding proteins (Derman et al., 1993; Prinz et al., 1997). Similarly, the absence of the reductant glutathione in the ER (Cuozzo and Kaiser, 1999) could allow air oxidation of protein disulfide bonds in the ER. Regardless, the O_2-FAD-Ero1p-PDI oxidase system examined here is the central mechanism by which eukaryotic cells promote disulfide formation.

Experimental Procedures

Yeast Strains and Methods

All yeast strains used were derived from a W303-1A parent. Yeast manipulations were performed using standard methods (Sherman, 1991). The strain background in the synthetic lethal screen contained an additional deletion of *ADE3* and was cir^o. Deletions of *ERO1*, *GSH1*, *ERV2*, and *FMO1* (YHR176W) were constructed using heterologous gene replacement (Longtine et al., 1998). Integrations of the UPRE-GFP (GFP driven by the unfolded protein response element) reporter (Travers et al., 2000) and pADH1-*FAD1* (*FAD1* overexpressed from the *ADH1* promoter) were performed by linearizing a pRS300-series plasmid (Sikorski and Hieter, 1989) and then targeted integration into the respective marker locus (*TRP1* or *HIS3*). Yeast media was supplemented with 0.5% Tween-80, an oleic acid derivative, and 20 μg/mL ergosterol for anaerobic growth. Anaerobic growth and experiments were performed in a controlled atmosphere chamber (Plas Lab) filled with an anaerobic gas mixture consisting of 85% N_2, 5% CO_2, and 10% H_2.

Plasmid Construction

The CEN/ARS (pRS315-based) plasmid (Sikorski and Hieter, 1989) overexpressing *FAD1* from the inducible *GAL1* promoter, and the 2μ plasmid (pRS425-based) overexpressing *ERO1*-zz from the inducible *GAL1* promoter (pBT006) were described previously (Tu et al., 2000). *FAD1* and *ERV2* were amplified from yeast genomic DNA using PCR and cloned into a pRS315-based vector for constituitive expression from the *ADH1* promoter (*FAD1*) or galactose-inducible expression from the *GAL1* promoter (*ERV2*).

Protein Purification

A plasmid for bacterial expression of a mutant PDI in which the first CxxC active site is changed to AxxA (PDI $[AxxA]_1$) was constructed from plasmid pBT101 (Tu et al., 2000) using Quikchange site-directed mutagenesis (Stratagene). Purified Ero1p, PDI, and reduced RNase A were prepared as described previously (Tu et al., 2000). The concentration of PDI was determined by A_{280} in 6 M guanidine hydrochloride and the concentration of Ero1p was determined using a Coomassie-based protein assay reagent (Bio-Rad).

Synthetic Lethal Screen

A synthetic lethal screen based on red/white colony sectoring was conducted to identify mutations synthetically lethal/sick with *ero1-1*. Briefly, a W303 Δ*ade3* strain harboring the *ero1-1* allele and a UPRE-GFP reporter was transformed with a *URA3*-marked pRS316 plasmid containing a wild type copy of *ERO1* and *ADE3*. On non-selective low adenine media, this strain gave rise to red colonies with white sectors. This strain was mutagenized using EMS at ~40% kill rate. The mutagenized pool was grown to log phase in selective media, treated with 0.5 mM DTT for 1 h, and then FACS sorted to collect the top 10-15% GFP-expressing cells to select for mutants with an intact or exaggerated UPR. Out of 20,000 cells that were plated on non-selective media, seven robust non-sectoring candidates were identified. Two of these were mutations in the *ERO1* locus. The remaining five candidates were recessive and showed 2:2 segregation, retained the plasmid for *ERO1,* and were not mutations in the chromomal *ERO1* locus. Each of these mutants was backcrossed to the parent, unmutagenized strain at least twice. Four of the five mutants were cloned by complementation using a pRS315-based yeast

genomic library of average insert size ~5 kb (D. Kellogg). One mutant was complemented by multiple plasmids each containing the *FAD1* locus. The *FAD1* gene of this mutant was sequenced and confirmed to contain a mutation of conserved G220 to S; this allele was termed *fad1-1*.

Analysis of Cellular Flavin Levels

Yeast (W303 Δ*ade3* background) were grown in SD media at 30°C to mid-late log phase (OD ~ 0.8) or stationary phase (OD ≥ 1.4). Approximately 10 ODs of cells were spun down, washed with H_2O, and resuspended in 20 mM Tris•Cl pH 7.9, 50 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 μM leupeptin, and 1 μM pepstatin A. The cells were lysed using glass beads, spun at 20,000 g for 15 min at 4°C, and then the supernatant was passed through a microcon YM-10 10 kDa filter to separate free, weakly-associated flavins from protein-bound flavins. Both the filtrate (free) and retentate (protein-bound) samples were analyzed for flavin content using reverse-phase HPLC as described previously (Tu et al., 2000).

Synthesis of Azido-FAD (FAD-2-N_3[β-^{33}P])

FAD synthetase from *Corynebacterium ammoniagenes* is capable of synthesizing FAD directly from riboflavin (Manstein and Pai, 1986). The gene was amplified from genomic DNA (ATCC) using PCR and subcloned into pBT101 for His_6-tagged overexpression in *E. coli* (Tu et al., 2000), and the FAD synthetase was purified using Ni-NTA agarose. The synthesis of azido-FAD was carried out in two steps. The first step involved conversion of riboflavin to FMN[^{33}P]: riboflavin (45 nmol) was incubated with an equimolar amount of ATP/ATP[γ-^{33}P] in 50 mM Tris•Cl pH 7.9 in the presence of ~10 μg purified *C.*

ammoniagenes FAD synthetase for a target specific activity of ~6000 Ci/mol FMN[^{33}P]. After 3 h at room temperature in the dark, a 10-fold molar excess (450 nmol) of 2-N_3-ATP (2-azido-adenosinetriphosphate, Affinity Labeling Technologies), 10 mM $MgCl_2$, and additional FAD synthetase (~10 μg) was added to drive conversion to the final azido-FAD product. After 20 h, the product was separated using reverse-phase HPLC and an acetonitrile/TFA gradient (final yield ~33% mol/mol riboflavin). To test specificity of the crosslinker, purified Ero1p (1.5 μM) and BSA (1.5 μM) were incubated with 10 μM azido-FAD in 10 mM HEPES pH 7.5, 50 mM NaCl, 0.05% digitonin, at room remperature for 30 min. Excess unbound azido-FAD was removed using a centrisep spun column (Princeton Separations). The azido-FAD crosslinker was activated using a handheld shortwave UV lamp (254 nm) for 1 min and then quenched with excess DTT (50 mM) on ice, and crosslinked proteins were analyzed by SDS-PAGE followed by phosphorimaging.

FAD Entry into Microsomes

ER-derived microsomes were prepared from wild type or Ero1p-zz overexpressing strains as described previously (Tu et al., 2000). Microsomes were incubated at room temperature for 10 min with or without treatment with 20 μg/mL Proteinase K in the absence or presence of 1% Triton X-100 in Buffer 88 (Brodsky et al., 1993) and then quenched with 2 mM PMSF. The microsomes were then resuspended in Buffer 88, 2 mM PMSF, and 10 μM azido-FAD. After 15 min at room temperature, the microsomes were washed and resuspended in Buffer 88, exposed to UV for 1 min, and quenched with 30 mM DTT. Microsomal proteins were separated by reducing SDS-

PAGE and analyzed by phosphorimaging, western blotting (using the F_c of IgG), and Coomassie staining.

RNase A Reoxidation Assays

The reoxidation of reduced RNase A under anaerobic conditions was carried out in an anaerobic chamber using degassed buffers. PDI was reduced using 10 mM DTT and buffer exchanged using a Centrisep spun column (Princeton Separations) into 20 mM sodium phosphate pH 8.0, 50 mM NaCl, and Ero1p was buffer exchanged into the same buffer but with 0.05% digitonin before addition. The final reaction buffer contained 50 mM sodium phosphate pH 8.0, 65 mM NaCl, 0.005% digitonin, PDI (1.7 μM), reduced RNase A (25 μM) and the indicated concentration of FAD and/or Ero1p (0.2 μM). After the indicated time points at 30°C, free thiols were blocked by the addition of SDS loading buffer and 10 mM AMS (4-acetamido-4'-maleimidylstilbene-2,2'-disulfonic acid, Molecular Probes). The oxidized and reduced forms of RNase A were detected by non-reducing SDS-PAGE followed by Coomassie staining. The recovery of RNase A activity was assayed as described previously (Tu et al., 2000) but with PDI (1.3 μM), Ero1p (0.2 μM), reduced RNase A (12.5 μM), and the indicated concentrations of FAD (0-50 μM).

Oxidation of PDI

Purified Ero1p (3 μM) was incubated with 10 μM FAD for 1 h at 4°C and then buffer exchanged into 20 mM HEPES pH 7.6, 100 mM NaCl, 1 mM EDTA, 0.05% digitonin using a Centrisep spun column to remove unbound FAD. We confirmed that virtually no free FAD comes through the buffer exchange process. Approximately 50% of Ero1p contained bound FAD by HPLC analysis. Reduced PDI $[AxxA]_1$ was prepared

by incubation in 10 mM DTT for 30 min at room temperature followed by buffer exchange into 20 mM HEPES pH 7.6, 50 mM NaCl, 2 mM EDTA. Various amounts of FAD-bound Ero1p were then added to a constant amount of reduced PDI $[AxxA]_1$ (2.0 μM). Oxidation of PDI was allowed to proceed for 5 min at and then quenched by addition of SDS Loading Buffer and 10 mM AMS, or pulsed with 10 μM FAD and oxidation allowed to proceed for an additional 7.5 min before quenching with AMS. Oxidized and reduced PDI were then detected by non-reducing SDS-PAGE followed by Coomassie staining. Doubling the total concentration of PDI and Ero1p while maintaining the same PDI:FAD-Ero1p ratios produced similar results.

Oxygen Consumption Assays

Dissolved O_2 levels were detected using a FO_2ton Fiber Optic Oxygen Sensing System, USB2000 spectrometer, and FOXY-AL300 Aluminum-jacketed Fiber Optic Probe (Harvard Apparatus). Catalysis of RNase A (60 μM) reoxidation was performed in air-saturated 0.1 M sodium phosphate pH 8.0, 50 mM NaCl, 0.01% digitonin in the absence or presence of Ero1p (0.5 μM), reduced PDI (3.5 μM), and FAD (50 μM). The reoxidation reaction was carried out at room temperature in a foil-wrapped, sealed vial, with the probe thread through a syringe needle into the vial to minimize atmospheric mixing. O_2 levels were recorded continuously for 30 min.

Detection of Hydrogen Peroxide, Effects of Catalase and Superoxide Dismutase

Peroxidase-catalyzed oxidation of o-dianisidine was used to monitor hydrogen peroxide levels during Ero1p-catalyzed oxidation. Reduced RNase A (25 μM) was added

to 0.1 M sodium phosphate pH 8.0, 50 mM NaCl, 0.01% digitonin, FAD (10 μM), PDI (2.5 μM), Ero1p (0.3 μM), 750 μM o-dianisidine, and 7.5 units horseradish peroxidase (Type X, Sigma) to initiate oxidation. A_{460} was monitored for 30 min at room temperature to follow H_2O_2 production. As a control, H_2O_2 (100 μM) was added to a reduced RNase solution to determine signal strength if yield of one H_2O_2/disulfide were observed. To determine the effects of catalase and superoxide dismutase on Ero1p-mediated oxidation, RNase reoxidation assays were performed as described previously (Tu et al., 2000) but with Ero1p (0.25 μM), PDI (1.7 μM), FAD (10 μM), reduced RNase A (15 μM), and catalase or superoxide dismutase (20 U/mL, Sigma) as indicated. Control experiments establish that the amount of catalase added to the reactions was sufficient to prevent virtually all oxidation of RNase A by stoichiometric amounts of H_2O_2.

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Footnotes

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SUPPLEMENT

Unpublished Data and Oxidative Folding in Mitochondria

During my rotation in the Weissman Lab, I did a screen for Snyder library insertions that are synthetically lethal with the *ero1-2* mutation. I found an insertion in *BLM3* that displayed a synthetic phenotype with *ero1-2*. However, I noticed the starting *ero1-2* strain seemed unusually sick, and questioned the validity of the synthetic phenotype. I later confirmed that a *ero1-2*Δ*blm3* strain grew normally. However, this led to the serendipitous finding that *ero1-1* mutant strains are highly sensitive to the antitumor antibiotic phleomycin at 0.5 μg/mL on YEPD, whereas Δ*blm3* strains which are reported to be sensitive grew normally. Phleomycin is a more water-soluble derivative of bleomycin, and they are thought to act as free radical DNA-cleaving agents (Mir et al., 1996). Bleomycin-iron complexes can activate molecular oxygen and the subsequent production of free radicals. Since the cellular environment of *ero1-1* strains is more reducing, the phleomycin-metal complex is perhaps more easily reactivated for multiple rounds of radical production. However, the absence of the reductant glutathione does not seem to suppress the phleomycin sensitivity of *ero1-1*.

I then reconstructed the *ero1-2ade2*Δ*ade3* strain and redid the synthetic lethal screen using EMS mutagenesis. Of 5,500 colonies screened, 6 candidates were non-sectoring, 5-FOA sensitive, and retained the plasmid because of *ERO1*. Two of the 6 candidates were not mutations in the *ERO1* gene itself, however, these mutants were not cloned. The 4 mutants that contained mutations in *ERO1* that led to synthetic lethality with the *ero1-2* mutation (H231Y) were: W497stop, C355Y, H237Y, and C355Y.

Upon purification of Ero1p-zz from yeast microsomes, treatment of the protein with AMS did not increase its apparent molecular weight during SDS-PAGE, indicating that most of its 14 cysteine residues are not in the reduced form and are likely to be in disulfide bonds (Figure 19). This is in agreement with what is observed with Ero1p in vivo (Frand, and Kaiser, 1999; Tu, B.P., unpublished data). Interestingly, in the *ero1-1* mutant, the ero1-1 protein exists in a more reduced form even at permissive temperatures (Ho-Schleyer, S.C., unpublished data). Co-expression of wild type Ero1p restores the ero1-1 protein to the oxidized form, suggesting that the oxidation state of the non-catalytic cysteines in Ero1p may be a reflection of the general redox environment in the ER.

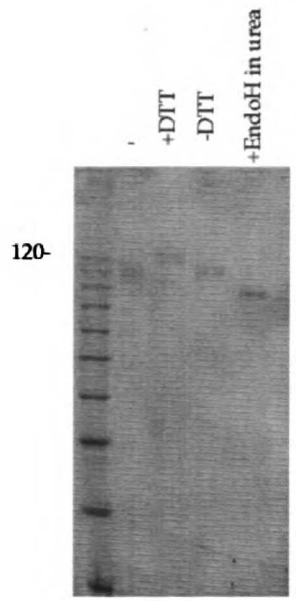

Figure 19. Purified Ero1p-zz from yeast microsomes is in a highly oxidized state
Lane 2 (–): -AMS, -DTT; lane 3 (+DTT): +AMS, +DTT; lane 4 (–DTT): +AMS, -DTT; lane 5 (EndoH): -AMS, +EndoH. Note that treatment of Ero1p-zz with AMS in the absence of DTT does not decrease its mobility.

The N-terminus of mature Ero1p begins at residue T19 (determined by Chris Turck). The signal sequence of Ero1p thus resides in amino acid residues 1-18.

Figure 20 shows the limited proteolysis pattern of purified Ero1p and BSA upon treatment with trypsin.

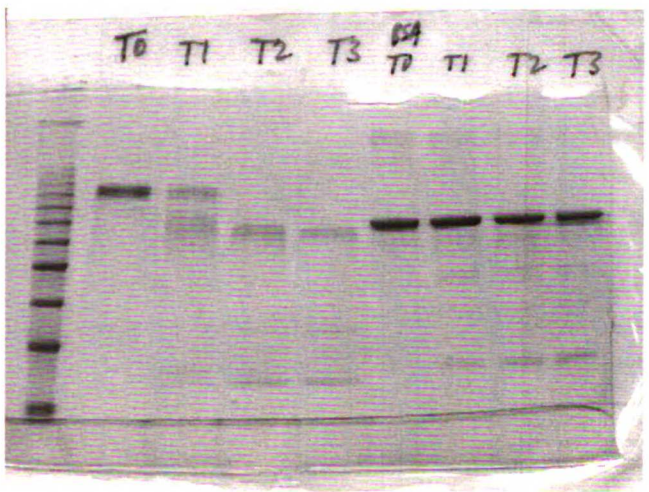

Figure 20. Limited proteolysis pattern of purified Ero1p
75 ng of trypsin was used to digest ~2 μg Ero1p. Proteolysis was terminated at the indicated time points: T1 = 35 min, T2 = 2h, T3 = 3h45.

Figure 21 shows the original wavelength scan of Ero1p taken using the Frankel Lab Aviv spectrophotometer.

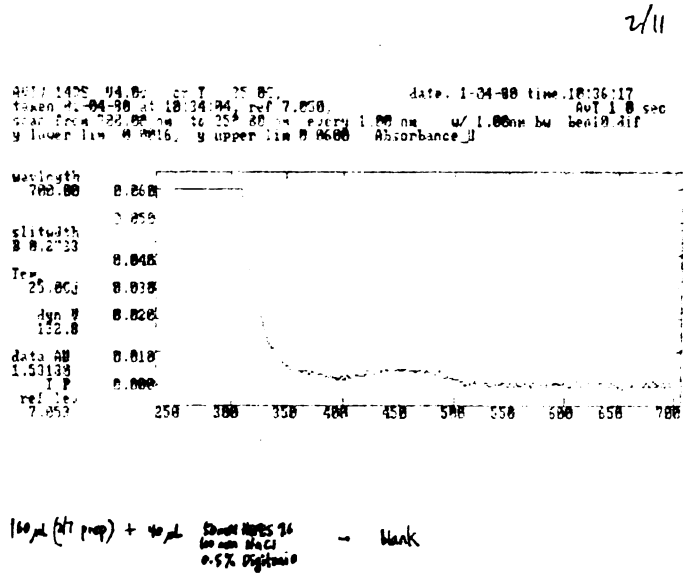

Figure 21. Wavelength scan of purified Ero1p
The scan was taken with ~50 μg protein using a 200 μL 1.0 cm path-length cuvette. Note the peak centered at 450 nm.

The Ero1p G229S (*ero1-1*) mutant protein exhibits very low activity in the RNase refolding assay even at room temperature. The Ero1p C352A mutant protein is inactive in the same assay. Ero1p that is purified from yeast microsomes does not seem to bind to ion exchange columns (MonoQ, S). It also does not bind to FAD-agarose beads (Sigma).

Under anaerobic conditions, purified Ero1p always exhibits a basal level of activity (measured by RNase A oxidation) that is dependent on FAD. This basal activity was observed regardless of the anaerobic conditions used (glove bag, the anaerobic

chamber, or in the presence of oxygen scrubbers). Furthermore, this level of activity is not improved by increasing free FAD levels 10-20 fold.

The yeast PDI homologs Mpd1p, Eug1p, and Mpd2p were cloned into bacterial vectors for recombinant overexpression. Mpd2p was insoluble under several conditions tested, but milligram quantities of Mpd1p and Eug1p were obtained. The ability of these homologs to interact with Ero1p in vitro was tested - Mpd1p (CGHC active site) for its ability to support Ero1p-mediated oxidation of RNase A, and Eug1p (CLHS, CIHS active sites) for its ability to act as a dominant inhibitor of Ero1p-mediated oxidation. Mpd1p could not support RNase A oxidation, and Ero1p did not seem to oxidize Mpd1p directly. Eug1p minimally inhibited Ero1p-mediated oxidation compared to the mutant PDI with CGHA active sites. These preliminary results suggest that Ero1p is a rather specific oxidant of PDI and does not interact with some of these PDI homologs. This would allow them to exist in a reduced form to assist in disulfide isomerization or reduction (see also Introduction). However, the Kaiser lab reports that a mixed disulfide between Ero1p and a mutant Mpd2p with a CGHA active site can be immunoprecipitated from yeast when both proteins are overexpressed (Frand and Kaiser, 1999). We could not test whether Ero1p interacts with Mpd2p in vitro since Mpd2p is insoluble under conditions tested.

In a screen for mutations synthetically lethal with the *ero1-1* mutation, mutations in *PDI1*, *FAD1*, *GPI16*, and *HUT1* displayed strong synthetic phenotypes with *ero1-1* (Tu and Weissman, 2002). The actual mutations in these genes were sequenced: *pdi1-1* –

P106S, (a conserved proline in the a domain), *fad1-1* – G220S (a conserved glycine), *gpi16-1* - P208S (a conserved proline), *hut1-1* – A265T (behaves as a null).

The function of *GPI16* (YHR188w) was unknown at the time I cloned it, but shortly after it was characterized as part of the glycophosphatidylinositol (GPI) transamidase complex that attaches GPI anchors to proteins (Fraering et al., 2001). It appears that many proteins with GPI anchors function in the cell wall or membrane and contain disulfide bonds. Thus, a defect in the attachment of GPI anchors together with a defect in the oxidative folding system in the ER may be too much for yeast to overcome.

The function of *HUT1* is unknown, although it has homology to a human UDP-galactose transporter related protein (Nakanishi et al., 2001). It is a 7-pass transmembrane protein localized in the ER. It may function as some sort of transporter, and remains possible that it is an ER FAD-transporter. Interestingly, Δ*hut1* is synthetically lethal with *ero1-1*, but is not synthetically lethal with Δ*ire1*, suggesting that Hut1p functions in oxidative folding and not some more generalized ER folding process.

A summary of genetic interactions:

Δ*hut1* not synthetic with Δ*ire1*
gpi16-1 synthetic with Δ*ire1*
gpi16-1 not synthetic with Δ*hut1*
ero1-1 weak synthetic with *gaa1-1*
gpi16-1 synthetic with *gaa1-1*
ero1-1 not synthetic with *gpi8-1*
gpi16-1 not synthetic with *gpi8-1*
ero1-2 not synthetic with Δ*hut1* or *gpi16-1*

overexpression of *HUT1* or *GPI16* does not suppress *ero1-1*

Gaa1p and Gpi8p are other components of the GPI transamidase complex (Fraering et al., 2001). From the results of these crosses, it appears that Gpi16p does function in the GPI anchor attachment pathway and that yeast cannot cope with defects in both oxidative folding and GPI anchor attachment. Hut1p does not appear to function in GPI anchor attachment and seems to carry out some function specific to Ero1p.

A high copy suppressor screen of the anaerobic sensitivity of *ero1-1* was conducted in hopes of identifying anaerobic electron acceptors of Ero1p. Both a 2μ library and a CEN/ARS *GAL* cDNA library were transformed into *ero1-1*, plated on SD media supplemented with Tween and ergosterol, and incubated in an anaerobic chamber at 30° for several days. For technical reasons and potential problems with the libraries, the screen was not rigorously done and only the strongest suppressors were picked and then cloned. Nothing from the 2μ library came up, and from the GAL cDNA library, *ERO1* and *ERV2* were identified as suppressors of the *ero1-1* anaerobic sensitivity when overexpressed.

Upon mutating either one of the CGHC active sites of PDI to AGHA to simplify the analysis of PDI oxidation states by AMS modification, I discovered that only PDI with the second C-terminal CGHC active site (PDI $[AxxA]_1$) intact could support Ero1p-mediated oxidation of RNase A (Figure 22). Strikingly, PDI with only the N-terminal CGHC active site (PDI $[AxxA]_2$) was completely incapable of supporting Ero1p-mediated oxidation of RNase A (Figure 22).

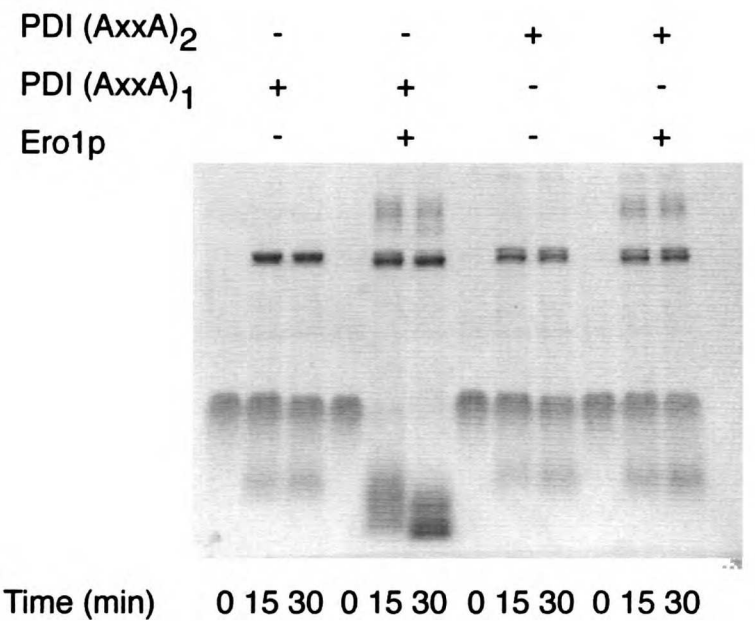

Figure 22. Ero1p oxidizes the second, C-terminal CGHC active site of PDI
Note that RNase A is converted to its oxidized from only in the presence of Ero1p and PDI with the second CGHC active site.

Thus Ero1p is a specific oxidant of the second CGHC active site of PDI that lies in the a' domain. This raises the question of the function of the first CGHC active site – perhaps it functions as a dedicated disulfide isomerase or reductase (see also Introduction).

Although deletions of *ERV2* and *FMO1* do not result in slower growth or increased sensitivity to DTT in either wild type or *ero1-1* strain backgrounds, it is still possible that the kinetics of oxidative folding in vivo could be affected in strains lacking these putative oxidases. It is very possible that disulfide formation in vivo can be compromised significantly before an effect on growth or sensitivity to DTT is observed. To test this possibility, the rates of CPY folding following DTT treatment were measured in strains lacking these putative oxidases (Figure 23).

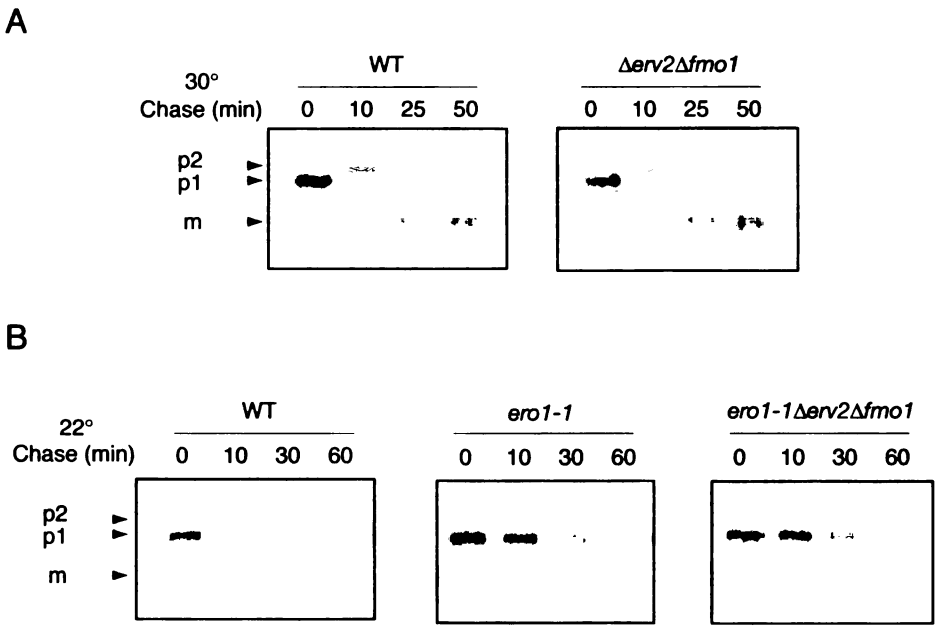

Figure 23. Oxidative folding of CPY in vivo is not compromised by the absence of Erv2p and Fmo1p in either a wild type or ero1-1 background
The indicated yeast strains were labeled with [^{35}S]methionine for 7 min in the presence of 5 mM DTT, and then chased in the absence of DTT for various time points at 30° or 22° as indicated. CPY was immunoprecipitated from cell lysates and analyzed by SDS-PAGE and phosphorimaging. The ER (p1), Golgi (p2), and vacuolar mature (m) forms of CPY are indicated.

Deletions of *ERV2* and *FMO1* do not affect the kinetics of CPY folding, suggesting that these oxidases do not play a general role in oxidative folding in the ER.

Oxidative folding in mitochondria

A popular theory is that the mitochondria organelle in eukaryotes originated from bacteria. Mitochondria have inner and outer membranes much like bacteria and the mitochondrial intermembrane space (IMS) is equivalent to the bacterial periplasm. Since bacterial oxidative folding occurs in the oxidizing environment of the periplasm, an intriguing possibility is that some oxidative folding processes may occur in the IMS of mitochondria. Recent studies have shown that a conserved and essential sulfhydryl oxidase Erv1p resides in the IMS (Lange et al., 2001). Erv1p seems to be the only member of the ERV/ALR family that is essential, and it can catalyze the formation of disulfide bonds in proteins directly using an FAD cofactor and molecular oxygen (Lee et al., 2000). Furthermore, some of the mitochondrial TIM proteins which reside in the IMS have been proposed to contain disulfide bonds rather than coordinate zinc (Curran et al., 2002a; Curran et al., 2002b).

I have developed a method to rapidly determine which proteins of a population contain disulfide bonds (Figure 24). The method involves first purifying mitochondria from yeast grown to log phase using equilibrium centrifugation (Nunnari et al., 2002). The mitochondrial proteins are precipitated with 15% TCA, washed with acetone twice, and then resuspended in 0.1 M Tris•Cl pH 7.8, 1% SDS, and 5 mM N-ethylmaleimide (NEM) to block all free cysteine residues for 1 h+ at room temperature. The remaining NEM is removed using a spun column equilibrated in 0.1 M Tris•Cl pH 7.3, 0.1% SDS. Disulfides are then reduced by the addition of 5 mM TCEP and incubation at 65° for 3 min. A second sulfhydryl-specific agent 7-diethylamino-3-(4'-maleimidyl-phenyl)-4-methylcoumarin (CPM, a fluorescent probe) is then added at 1 mM to label all the

cysteines that were previously in disulfide bonds for 1 h at room temperature. After SDS-PAGE, the proteins that previously contained disulfide bonds will fluoresce upon UV excitation.

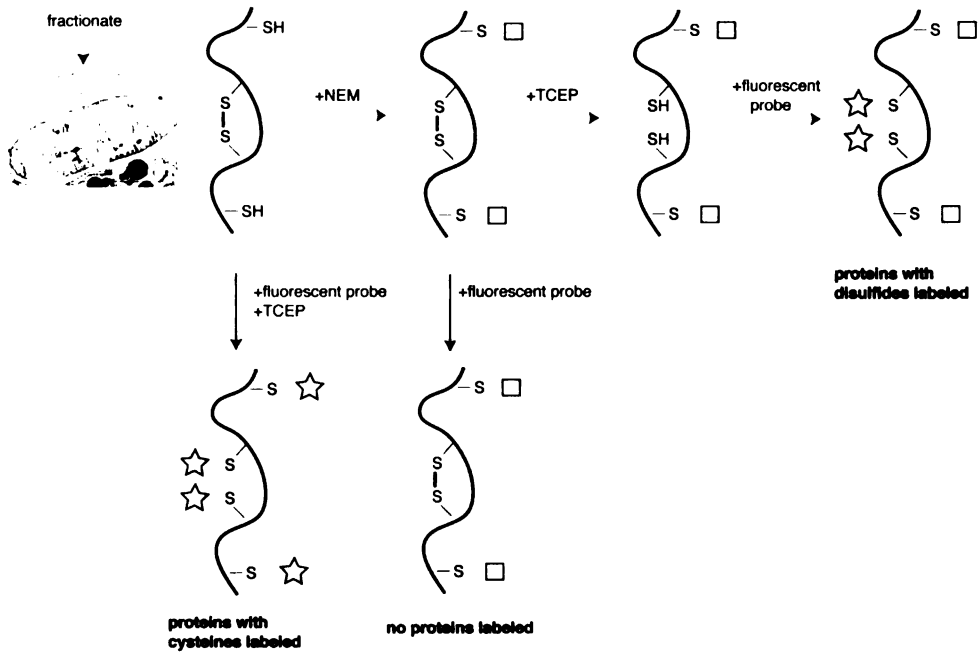

Figure 24. Scheme for identifying mitochondrial proteins with disulfide bonds

To identify these disulfide-containing proteins, the same method (Figure 24) can be used, but the second sulfhydryl-specific modification is performed with 1 mM N^{α}-(3-maleimidylpropionyl)biocytin (biotin-maleimide, a biotinylation reagent) instead of CPM for 1 h at room temperature. The unreacted biotin-maleimide is removed using a spun column equilibrated in 0.1 M Tris pH 7.3, 0.001% SDS, and then ~50 µL streptavidin-agarose beads washed with 50 mM Tris•Cl pH 7.3, 0.5 M NaCl, 0.5% Tween-20 are added to pull down the biotinylated proteins for 1 h+ at room temperature. The beads

are then washed with 5-10 mL of the above buffer, and then boiled in 50 mM Tris•Cl pH 6.8, 2% SDS, 0.4 M urea + 1X SDS Loading Buffer for 3 min to elute bound proteins. The proteins are then separated by SDS-PAGE and stained with silver.

Using this method, I determined that in a yeast cytosolic fraction, very few proteins are labeled which indicates virtually no disulfide-containing proteins are present (Figure 25). In an ER microsome fraction, I was able to confirm that many proteins are labeled indicating an abundance of disulfide-containing proteins in the ER. Interestingly, in a purified mitochondria fraction, a consistent set of proteins is labeled (~10-12 that can be visualized by eye). The labeled proteins are fewer in number and are distinct from those labeled in an ER fraction (Figure 25). These preliminary observations suggest that there are some disulfide-containing proteins present in mitochondria. I have now applied the biotin-labeling procedure to the mitochondrial proteins and have pulled down a subset of these putative disulfide-containing proteins. These proteins will be identified by mass spectrometry sequencing and further experiments will be designed accordingly. Preliminary experiments suggest there are no significant differences between the set of disulfide-containing mitochondrial proteins in an *erv1* temperature sensitive strain and a wild type strain after a shift to the non-permissive temperature 37° for 6 h.

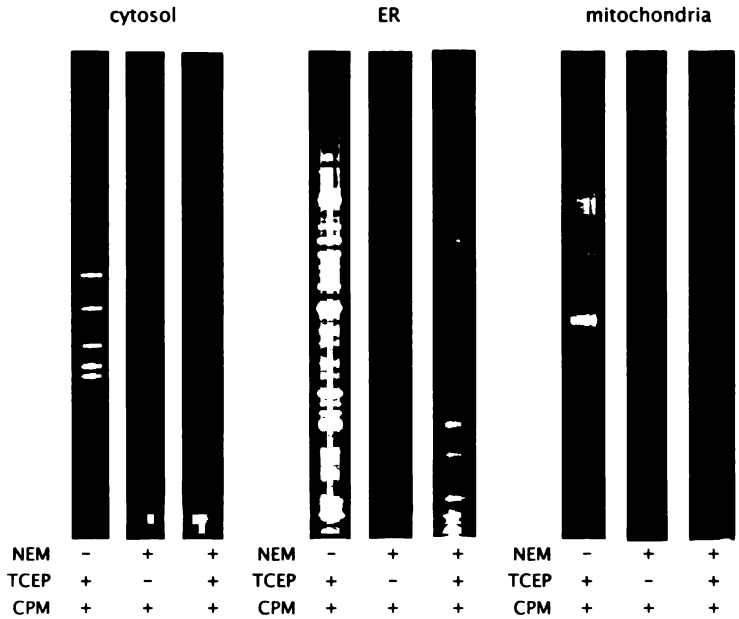

Figure 25. Determination of disulfide-containing proteins in cytosol, ER, and mitochondria

CHAPTER 4

Summary

In summary, while many previous studies of oxidative folding have focused on the bulk redox potential of the ER, it is now evident that eukaryotic disulfide formation proceeds by the kinetic shuttling of oxidizing equivalents to folding substrates. Since the Ero1p-driven oxidation machinery is insulated from the bulk redox environment, reduced glutathione and perhaps certain PDI homologs can assist in the isomerization and reduction of incorrect disulfide bonds. Oxidative folding is coupled to the strong reduction potential of molecular oxygen through a FAD-dependent mechanism, but a potential consequence is the production of toxic reactive oxygen species. Indeed, it appears that Ero1p could be a significant contributor to cellular oxidative stress, suggesting that its activity must be carefully adjusted according to the folding load on the ER.

Future work will determine how Ero1p is anchored to the ER membrane and the role of this membrane-association. Membrane-association and proximity to the translocon may be important to allow Ero1p to support co-translational disulfide formation. The precise mechanism of electron transfer from Ero1p to FAD to molecular oxygen still remains to be determined, and it is likely that the 7 conserved cysteine residues in Ero1p play an important role in this process. Of great interest is what happens to the molecular oxygen consumed by Ero1p and whether Ero1p can mediate the complete reduction of molecular oxygen to water. In addition, the anaerobic terminal electron acceptors for Ero1p remain unknown.

It is also unclear how Ero1p interacts specifically with PDI and not with its numerous homologs, despite their high degree of homology. In addition, the cellular role

of these PDI homologs - whether they function as dedicated isomerases or reductases, or act on specific folding substrates - remains a wide open question. Perhaps in eukaryotes there is a disulfide reductase system that transfers the reducing potential of the cytosol across the membrane to the ER lumen in a manner similar to the DsbD-DsbC system in bacteria. It has also become evident that the two active sites of PDI are not equivalent. Ero1p specifically oxidizes the second, C-terminal active site of PDI, perhaps allowing the first active site to function in disulfide isomerization or reduction.

Finally, since Ero1p activity can be a significant source of cellular oxidative stress, it is intriguing to determine whether controlling the levels of free FAD available to Ero1p is a physiologically significant mechanism to regulate its activity. How cellular levels of FAD are established is largely unknown, but preliminary experiments indicate that free FAD levels in yeast can vary according to its growth phase and conditions. There may also be other mechanisms that contribute to the regulation of oxidative folding. Future studies will reveal how the ER maintains its optimal environment for the multitude of redox processes required for the proper folding of secretory proteins.

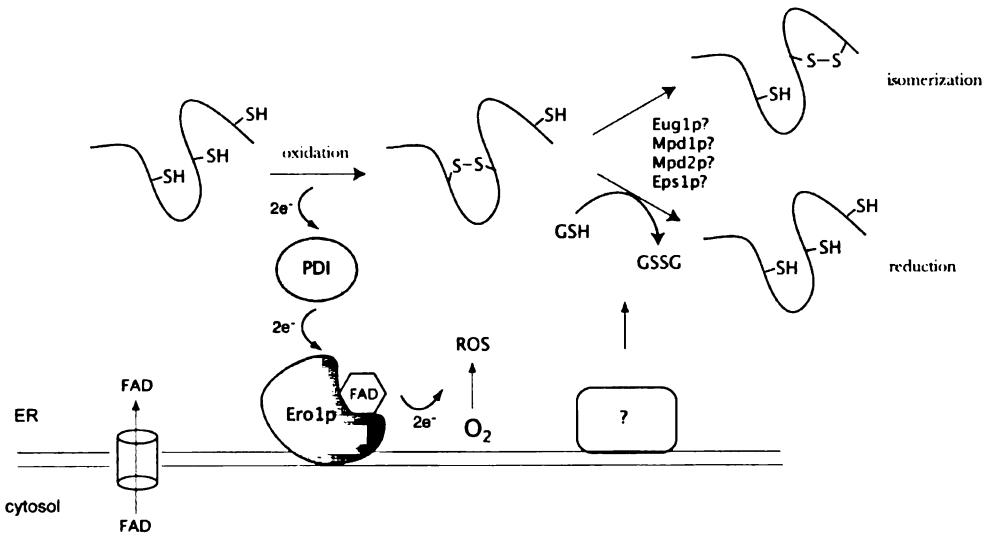

Figure 26. Oxidative protein folding in the ER (August 2003)

APPENDIX A

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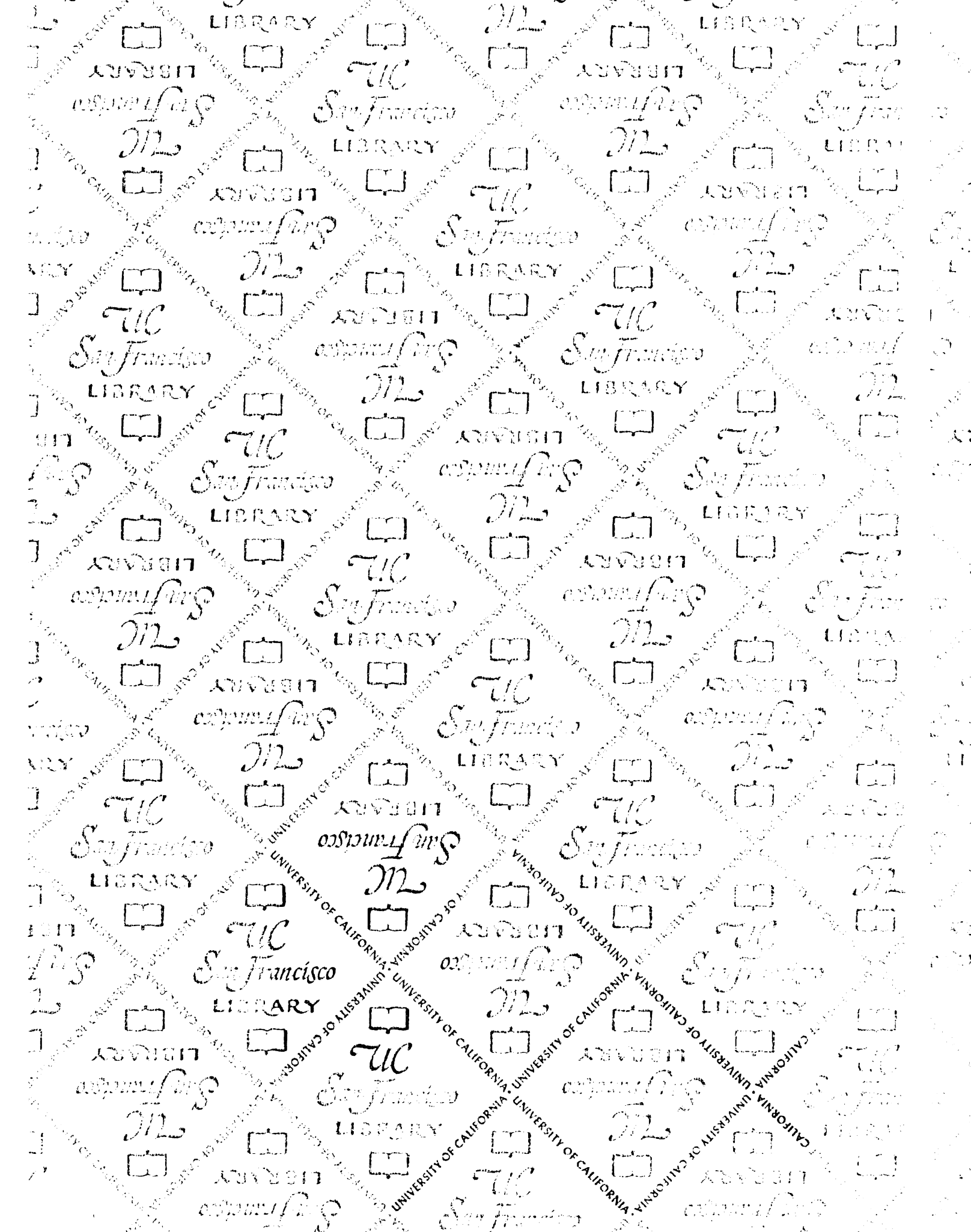
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