Disulfide bond catalysis is an essential component of protein biogenesis in the secretory pathway, from yeast through to man. In the endoplasmic reticulum (ER), protein-disulfide isomerase (PDI) catalyzes the oxidation and isomerization of disulfide bonds and is re-oxidized by an endoplasmic reticulum oxidoreductase (ERO). The elucidation of ERO function was greatly aided by the genetic analysis of two ero mutants, whose impairment results from point mutations in the FAD binding domain of the Ero protein. The ero1-1 and ero1-2 yeast strains have conditional and dithiothreitol-sensitive phenotypes, but the effects of the mutations on the behavior of Ero proteins has not been reported. Here, we show that these Gly to Ser and His to Tyr mutations do not prevent the dimerization of Ero1β or the non-covalent interaction of Ero1β with PDI. However, the Gly to Ser mutation abolishes disulfide-dependent PDI-Ero1β heterodimers. Both the Gly to Ser and His to Tyr mutations make Ero1β susceptible to misoxidation and aggregation, particularly during a temperature or redox stress. We conclude that the Ero FAD binding domain is critical for conformational stability, allowing Ero proteins to withstand stress conditions that cause client proteins to misfold.

Endoplasmic reticulum oxidoreductases (Eros) are required for the provision of disulfide bonds in the endoplasmic reticulum (ER) (1). The control of disulfide bond formation is an integral part of the quality control machinery of the secretory pathway, with disulfide bonds being used for structural stability, enzyme activity, or regulation of various native proteins. Often, ER protein misfolding is a component of disease pathology. In cystic fibrosis, for example, the ΔF508 mutation of cystic fibrosis transmembrane conductance regulator severely compromises the stability of the nascent polypeptide at the ER, resulting in the eventual degradation of the protein in the cytosol by the proteasome (2). Neurological disorders such as Alzheimer Disease, Huntington Chorea, and Creutzfeldt-Jakob disease all have an ER misfolding or stress component, and in amyloidosis, the cell specific ER environment and folding “signature” can determine whether a protein is appropriately secreted (3).

The ER contains a number of chaperones and folding factors to guide and monitor the status of its client proteins (4). The lectin-like chaperones calnexin and calreticulin oversee glycoprotein folding, in concert with the PDI homolog ERP57 (5). Calnexin and calreticulin work together with glucosidases and UDP-glucose:glycoprotein glucosyltransferase to ensure that only properly folded glycoproteins exit the ER (6). With calnexin and calreticulin present to ensure proper folding of glycoproteins, the hsp70 chaperone BiP (GRP78) and PDI may preferentially fold proteins with no or less accessible glycans (7). Misfolded luminal proteins are usually destroyed by an ER-associated degradation pathway, which involves recognition of an unfolded protein by the lectin-like EDEM protein(s), expulsion from the ER via Sec61 or p97/VCP, and degradation in the cytosol by the ubiquitin-proteasome system (8). If ER-associated degradation is unsuccessful, misfolded proteins in the ER can lead to accumulation of reactive oxygen species and cell death (9).

Protein folding in the ER requires an oxidising environment for the generation of disulfide bonds (10). The redox peptide glutathione is important in buffering the ER (11), regulating the rate of ER protein oxidation (12, 13), and providing reducing equivalents for reductive/isomerization pathways (14). PDI is the major enzyme responsible for ER protein oxidation (15). In Saccharomyces cerevisiae, PDI is maintained in the oxidized state by another protein, the oxidoreductase Ero1p, which is essential for viability (16–18). Whereas S. cerevisiae employs one ERO gene, higher organisms have two ERO genes. In mammals, Ero1α is induced by hypoxia (19) and may play a role in ER degradation (20), whereas Ero1β is induced by the unfolded protein response (21), with Ero1β showing high expression levels in selected secretory tissues (22).

Eros donate disulfide bond equivalents asymmetrically to the two redox-active a and a’ thioredoxin domains of PDI (23) and in turn receive electrons that are passed on to O2 (24) and probably other electron acceptors including free FAD (25). Eros have two cysteine-rich sites that are important for electron transfer and oxidation (26, 27): an N-terminal CxxxCx site, which transfers oxidising equivalents to PDI, and a C-terminal
The ero1-1 mutation results in temperature-dependent lethality, whereas the ero1-2 mutation results in hypersensitivity to reducing agents (DTT) (16, 17). Single amino acid substitutions in Ero1p cause the ero1-1 and ero1-2 phenotypes. These mutations are G229S (equivalent to Gly252 in Ero1β) and H231Y (equivalent to His254 in Ero1β). Both residues are within the Ero flavin fold, with His231 directly contacting the ribose 5′-phosphate group of the FAD moiety (29). In Ero1p, residues Arg187, Thr189, Trp200, Ser228, His231, and Arg260 form hydrogen bonds or salt bridges with the FAD cofactor and are conserved in Ero1α and -β. The alteration of structure and charge within the flavin fold of the ero1 mutants is therefore likely to lead to loss of function by preventing electron transfer from occurring normally. However, the biochemistry of the FAD binding site mutants has not been fully examined.

Using Ero1β, we show that FAD domain mutants misoxidise during redox or temperature stress. The unusual Ero FAD binding domain therefore has a dual function: to facilitate electron transfer and to confer protein stability. The flavin pocket safeguards protein oxidation pathways, preventing spurious electron transfer at times when client proteins will misfold, misoxidise or aggregate.

**EXPERIMENTAL PROCEDURES**

**Cell Lines and Antibodies**—Human cervical carcinoma HeLa cells were maintained in minimal essential medium (Invitrogen) supplemented with 8% fetal bovine serum (Sigma), 2 mM Glutamax, 100 units/ml penicillin, and 100 μg/ml streptomycin (Invitrogen) at 5% CO2. The polyclonal rabbit anti-sera against PDI (28) and Ero1β (Q86YB8). * indicates the conserved glycine and histidine residues that are mutated in the ero1-1 and ero1-2 yeast strains. B and C, post-nuclear supernatants from HeLa cells transfected with wtEro1β-Myc (lanes 1–4), Ero1βH254Y-Myc (lanes 5–8), Ero1βG252S-Myc (lanes 9–12), and mock (lanes 13 and 14) were incubated with 0, 0.25, 1.25, and 2.5 μg/ml TPCK-treated trypsin for 30 min at 4 °C, analyzed by reducing SDS-PAGE, and immunoblotted with αEro1β (B) and αMyc (C). Generated fragments are labeled A and B.

**Western Blotting**—Cells were lysed in lysis buffer supplemented with 20 mM N-ethylmaleimide or 20 mM iodoacetate to trap disulfide bonds. Nuclei were removed by centrifugation at 16,000 × g for 10 min at 4 °C. Post-nuclear cell lysates or immunoprecipitates were analyzed by SDS-PAGE. Immunoprecipitations were carried out using 8 μl of αPDI or 0.5 μl of αHA (HA-7) or αMyc (9B11) antibodies immobilized on 50 μl of a 20% suspension of Protein A-Sepharose beads, followed by washing twice with lysis buffer. Proteins were transferred to polyvinylidene difluoride membranes (Millipore) at 150 mA for 2 h or 30 V overnight, washed extensively, and visualized by ECL (GE Healthcare/Amersham Biosciences) upon exposure to Biomax Light film (Eastman Kodak Co.). Protein markers were from Bio-Rad. Each blotting experiment was reproduced at least twice.

**DTT and Temperature Treatments**—Transfected HeLa cells were washed with PBS (Invitrogen) and incubated with complete medium containing 10 mM DTT (Sigma) or buffer only for 15 min at 37 °C. For temperature treatments, transfected HeLa cells were incubated at 24, 37, and 42 °C on water baths for 1 h in complete medium buffered with 10 mM HEPES, pH 7.4 (Invitrogen). Cells were washed and lysed as described above,
and post-nuclear supernatants were subjected to analysis by 8\% SDS-PAGE.

Trypsin Sensitivity Assay—Post-nuclear supernatants were incubated with 0, 0.25, 1.25, and 2.5 \mu g/ml TPCK-treated trypsin (Sigma) for 30 min at 4°C. Proteolytic digestion was terminated by the addition of 200 \mu g/ml soybean trypsin inhibitor, in addition to 10 \mu g/ml each of chymostatin, leupeptin, antipain, and pepstatin A. Samples were taken up into sample buffer and analyzed by 10\% SDS-PAGE. Five independent trypsin assays were performed.

RESULTS AND DISCUSSION

Limited Proteolysis of Ero1βG252S and Ero1βH254Y—We have previously characterized the human Ero1β protein and shown that Ero1β homodimerises and interacts with PDI by both non-covalent and covalent (disulfide-dependent) interactions (22). Ero1β is functionally equivalent to Ero1p (21), and both Ero1β and Ero1p bind to PDI (31) and homodimerise (32). Since Ero1p and Ero1β also share conserved FAD binding residues (Fig. 1A), we used our knowledge of Ero1β to study the effects of the ero1-1 and ero1-2 mutations on Ero gene products. We undertook site-directed mutagenesis to create tagged Ero1βG252S and Ero1βH254Y mutants. Myc- and HA-tagged human Eros are functional, reside in the ER, and can interact with PDI in the same way as their non-tagged counterparts (21, 22, 28, 33).

We found that biosynthesis of Ero1β was largely unaffected by the G252S and H254Y mutations in the FAD domain (supplemental Fig. 1). However, it was possible that mutations in this region could cause conformational changes resulting in misfolding, particularly since Ero1p seems to expose buried residues during the oxidation cycle (32). To compare the overall conformation of wild-type Ero1β and the mutant proteins, we used a limited proteolysis approach. Partial trypsin digestion has been used to map conformational changes in a number of ER proteins, including polyomavirus (34) and cystic fibrosis transmembrane conductance regulator (35, 36). Thus transfected HeLa cell post-nuclear supernatants were treated with a concentration range of TPCK-trypsin for 30 min on ice. The reaction was quenched with soybean trypsin inhibitor and the lysates were analyzed by reducing SDS-PAGE, Western blotting, and detection with either αMyc or αEro1β. Oxidized (OX) and reduced (R) Ero1β monomers as well as co-immunoprecipitated Ero1β are indicated.

![Figure 2. Ero1βG252S and Ero1βH254Y interactions with PDI. Lysates from HeLa cells transfected with Ero1βG252S-Myc (A) and Ero1βH254Y-Myc (B) were analyzed directly by immunoblotting with αMyc after non-reducing and reducing SDS-PAGE (lanes 1–5) or after immunoprecipitation with αPDI (lanes 6–9/10). C, lysates from HeLa cells transfected with wtEro1β-Myc (lanes 4, 8, and 13), Ero1βG252S-Myc (lanes 1, 5, and 10), Ero1βH254Y-Myc (lanes 2, 6, and 11), and Ero1βC390A-Myc (lanes 3, 7, and 12) were immunoblotted with αMyc before (lanes 1–4) or after (lanes 5–13) immunoprecipitation with αPDI. Lane 5 from A, lanes 3 and 8 from B, and lane 9 from C are loaded with sample buffer only. αMyc cross-reactive antibody. Disulfide-dependent complexes are shown as *.

Oxidized (OX) and reduced (R) Ero1β monomers as well as co-immunoprecipitated Ero1β are indicated.](25020/images/25020fig2.jpg)
Tyr\textsuperscript{329} to Leu\textsuperscript{343}), the use of these antibodies can provide some positional information about the fragments.

In each of five experiments, wild-type Ero1β-Myc was digested into two major fragments (Fig. 1). The largest fragment of \( \sim 55 \text{kDa} \) appeared with 1.25 \( \mu \text{g/ml} \) trypsin (fragment A, Fig. 1B, lane 3). This fragment must contain at least the (Cxx)CxxC motif and the C-terminal Myc tag (Fig. 1C, lane 3) and is therefore likely to have lost the N terminus of the protein. The smaller product (fragment B, Fig. 1B, lane 4) was an \( \sim 45\text{-kDa} \) species that lacked the C-terminal Myc tag (absent from Fig. 1C, lane 4) but is likely to contain the (Cxx)CxxC motif based on size and the presence of the Tyr\textsuperscript{329}–Leu\textsuperscript{343} epitope. Given the sizes of the fragments generated and the clustering of lysine residues in an exposed loop in this region, the results suggest that Ero1β was selectively proteolysed between the two redox-active domains. Furthermore, a trypsin digestion prediction suggests that Ero1p is also likely to be cleaved in this region.

The digestion patterns of the H254Y mutant (Fig. 1, B and C, lanes 5–8) and the G252S mutant (Fig. 1, B and C, lanes 9–12) and their overall sensitivity to trypsin were comparable with wild-type Ero1β, suggesting that the gross conformation of each protein was similar. However, there were some differences between the substrates, such as the appearance of a shadow band under the full-length protein in the mutant digestions probed with \( \alpha \text{Myc} \) (Fig. 1C, lanes 7, 8, 11, and 12) and the lower abundance of fragment B in the G252S digestions (Fig. 1B, lane 12). The H254Y and, in particular, G252S mutation may have localized effects upon Ero structure and folding. However, given the overall similarity of the digestion product sizes, we conclude that gross conformational change and increased lysine exposure is unlikely to result from point mutations in the FAD binding site under normal steady state conditions.

**Differences in the Covalent and Non-covalent Interactions of Ero1βG252S and Ero1βH254Y with PDI**—Having shown that the G252S and H254Y mutations did not cause extreme structural changes, we investigated the PDI binding properties of the mutants. Previously, we have shown that wild-type Ero1α and Ero1β both form intermolecular disulfide bonds with PDI (covalent interactions) and both co-immunoprecipitate with PDI (representing a combination of covalent and non-covalent interactions) (22, 28). Upon non-reducing SDS-PAGE, Ero1β resolves as a monomer and as a collection of disulfide-bonded forms that include PDI-Ero1β and Ero1β-Ero1β complexes (22).

Thus HeLa cells were transfected with either G252S (Fig. 2A) or H254Y (Fig. 2B), and the post-nuclear supernatants were analyzed by reducing and non-reducing SDS-PAGE. Under non-reducing conditions, G252S ran as a monomeric population (OX) and as a collection of trapped disulfide-bonded species (Fig. 2A, lane 1), which disappeared upon reduction of the samples with DTT (Fig. 2A, lane 3). Note the background band that was also present in the mock transfectant (Fig. 2A, lanes 2 and 4). G252S specifically co-immunoprecipitated with the PDI antiserum (Fig. 2A, lanes 8 and 9), but disulfide-dependent Ero-PDI dimers were not evident when compared with the background (antibody) bands from the mock transfectant (Fig. 2A, lanes 6 and 7). Like G252S, the H254Y mutant also ran as a monomer, but the trapped, higher molecular weight species were more abundant and less diffuse than seen with G252S (Fig. 2B, lane 1, compare with Fig. 2A, lane 1). H254Y specifically co-immunoprecipitated with PDI (Fig. 2B, lanes 9 and 10), but unlike G252S, H254Y became trapped in a disulfide-dependent complex with PDI under non-reducing conditions (compare Fig. 2B, lanes 6 and 7 with Fig. 2A, lanes 6 and 7).

We compared the two mutants directly alongside wild-type Ero1β and an active site mutant with altered PDI binding properties (Ero1βC390A-Myc). Cell lysates were directly analyzed
by non-reducing SDS-PAGE, blotted, and probed with αMyc (Fig. 2C, lanes 1–4). Equal portions of the lysates were subjected to immunoprecipitation with αPDI followed by blotting with αMyc under reducing (Fig. 2C, lanes 5–8) or non-reducing conditions (Fig. 2C, lanes 10–13). All Ero1β proteins resolved as a collection of monomeric and disulfide-linked forms (Fig. 2C, lanes 1–4). G252S, H254Y, C390A, and wild-type Ero1β could all be co-immunoprecipitated with PDI (Fig. 2C, lanes 5–8). However, only H254Y (Fig. 2C, lane 11) and wild-type (Fig. 2C, lane 13) formed prominent, discretely disulfide-dependent complexes with PDI. We conclude from this experiment that both G252S and H254Y can interact non-covalently with PDI but that inter-molecular disulfide bonding with PDI is disturbed in the G252S mutant.

Ero1β G252S and Ero1β H254Y Both Dimerize—Wild-type Ero1β can form homodimers, and mutating Cys396 of the Ero1β active site impedes both function and dimerization (22). Ero1β interactions may therefore be important for Ero1β regulation and/or activity (32). To ask whether ero1-1 and ero1-2 phenotypes could be partly explained by a failure of mutant Eros to self-complex, we investigated whether the G252S and H254Y mutants interacted with wild-type Ero1β. For this, we co-transfected HeLa cells with both HA and Myc-tagged versions of Ero1β. Since these tagged proteins have different molecular weights, they can be discriminated by their migration on SDS-PAGE gels.

Under reducing conditions, G252S-Myc (Fig. 3A, lane 1) migrated more slowly than wt-HA (Fig. 3A, lane 2), as expected. In double transfecants, both G252S-Myc and wt-HA could be detected (Fig. 3A, lane 3). No signal was seen in mock transfecants (Fig. 3A, lane 4). Lysates from these transfecants were subjected to immunoprecipitation with αHA and the blots probed with αMyc. Whereas no Ero1β signal could be detected from the single transfecants or mock lysates (Fig. 3B, lanes 1, 2, and 4), G252S-Myc clearly co-immunoprecipitated with HA-tagged wild-type Ero1β (Fig. 3B, lane 3). Similar results were obtained with the H254Y mutant: the single and double transfecants expressed the proteins expected (Fig. 3C, lanes 1–3) and the H254Y mutant co-immunoprecipitated with HA-tagged wild-type Ero1β (Fig. 3D, lane 3).

Having demonstrated that the G252S and H254Y mutants interacted with wild-type Ero1β, we investigated whether mutant-mutant interactions could occur. This was of interest because an Ero1βC396A mutant heterodimerizes with wild-type Eros but fails to form mutant-mutant dimers (22). Thus cells were transfected with individual HA-tagged mutants or co-transfected with both the Myc- and HA-tagged versions of the same mutant. The lysates were analyzed by SDS-PAGE to verify transfection and were subjected to immunoprecipitation with αMyc prior to immunoblotting with the anti-Ero1β serum.

The single and double transfecants expressed the expected proteins (Fig. 3E, lanes 1–4). No signal was observed in mock transfecants (Fig. 3E, lane 5). Upon immunoprecipitation with αMyc, no Ero1β signal was detected in the H254Y-HA, G252S-HA, or mock transfecants, as expected (Fig. 3E, lanes 7, 8, and 11, respectively). However, H254Y-HA clearly co-immunoprecipitated with H254Y-Myc (Fig. 3E, lane 9) and likewise G252S-HA was co-isolated with G252S-Myc (Fig. 3E, lane 10). We conclude from these experiments that point mutations in the FAD binding domain do not prevent Ero1β mutants from self-complexing or interacting with wild-type Ero1β molecules in the ER.

Aberrant Oxidation of Ero1βG252S and Ero1βH254Y during Reducing and Temperature Stress—The yeast counterparts of G252S and H254Y are hypersensitive to temperature stress and reductants (16, 17). We therefore examined the fate of the G252S and H254Y proteins when mammalian cells were subjected to similar conditions. First, we established the effects of DTT treatment on living HeLa cells transfected with wild-type Ero1β. We found that at steady state, 10 mM DTT in the medium eliminated the majority of disulfide-dependent (Ero-PDI and Ero-Ero) complexes when the lysates were subsequently analyzed by non-reducing SDS-PAGE (Fig. 4A, lanes 1 and 2). A similar result was obtained with cells co-transfected with wild-type Ero1β-HA and wild-type Ero1β-Myc (Fig. 4A, lanes 3 and 4). When the samples were analyzed by reducing SDS-PAGE, a single reduced band was recovered for each mutant, as expected (Fig. 4A, lanes 7–10). The small shift in mobility of Ero1β after exposing the cells to DTT was a consequence of increasing availability of -SH groups to the alkylating agent (Fig. 4A, compare lanes 7 and 8 and lanes 9 and 10).

To confirm that Ero1β complex formation was abolished by DTT treatment, lysates from the DTT-treated and untreated cells were subject to immunoprecipitation with αMyc and probed with αEro1β serum after immunoblotting. As expected, the wt-Myc protein from single transfecants was detected regardless of DTT treatment (Fig. 4B, lanes 1 and 2). Ero1β-HA only co-immunoprecipitated with Ero1β-Myc in the absence of DTT treatment (Fig. 4B, lane 4). We conclude from this experiment that a strongly reducing environment disrupts the majority of Ero1β-mediated covalent and non-covalent interactions at steady state.

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**FIGURE 4. Oxidative misfolding of Ero1βG252S and Ero1βH254Y.** A and B, HeLa cells transfected with wtEro1ββ-HA (lanes 1 and 2 and 7 and 8), wtEro1ββ-Myc plus wtEro1ββ-HA (lanes 3 and 4 and 9 and 10) and mock (lanes 5 and 6 and 11 and 12) were treated with 10 mM DTT for 15 min. Post-nuclear lysates were analyzed by immunoblotting with αMyc after non-reducing SDS-PAGE (lanes 1–6) and with αEro1β after reducing SDS-PAGE before (lanes 7–12) or after (B) immunoprecipitation with αEro1 β-Myc (lanes 1 and 2), Ero1βH254Y-Myc (lanes 3 and 4), Ero1βG252S-Myc (lanes 5 and 6), and mock (lanes 7 and 8) analyzed by immunoblotting with αMyc after non-reducing (C) and reducing (D) SDS-PAGE. Disulfide-dependent Ero-PDI and Ero-Ero complexes are denoted as *, with higher molecular weight complexes and aggregates as **. Oxidation states of Ero1β monomers are shown as OX (oxidized) and R (reduced). E, HeLa cells transfected with Ero1βH254Y-Myc (lanes 1–3) and mock (lanes 4–6) were treated with DTT as for A (lanes 2 and 3) and allowed to recover by washing out DTT for 30 min (lanes 3 and 6). Post-nuclear lysates were analyzed by immunoblotting with αMyc after non-reducing SDS-PAGE. F and G, HeLa cells transfected with wtEro1ββ-Myc (lanes 1–3), Ero1βH254Y-Myc (lanes 4–6), Ero1βG252S-Myc (lanes 7–9), and mock (lanes 10–12) were incubated at either 24, 37, or 42 °C for 1 h. Post-nuclear lysates were analyzed on non-reducing (F) and reducing (G) SDS-PAGE prior to immunoblotting with αMyc (F) or Ero1β (G). Disulfide-dependent Ero-PDI and Ero-Ero complexes are denoted as * with higher molecular weight complexes and aggregates as **. Oxidation states of the Ero1β monomer are shown as OX (oxidized) and R (reduced).
Next, we examined the fate of H254Y and G252S after incubating cells with DTT. Cell lysates from treated and untreated transfectants were examined by non-reducing SDS-PAGE and were immunoblotted with αMyc. Wild-type Ero1β became mostly reduced and lost virtually all detectable disulfide-dependent interactions, as seen in Fig. 4A (Fig. 4C, lanes 1 and 2). However, the H254Y and G252S mutants behaved differently. H254Y maintained its complexes in the face of a DTT challenge (Fig. 4C, lanes 3 and 4, *). For G252S, DTT treatment caused a reproducible increase in the proportion of higher molecular weight complexes and aggregates (Fig. 4C, lanes 5 and 6, **). As an internal control, monomeric Ero1β became reduced upon DTT treatment (R, Fig. 4C, lanes 1, 3, and 5). All Ero1β molecules could be reduced in vitro when DTT was added to the sample buffer (Fig. 4D, lanes 1–6), demonstrating that the species seen in the non-reducing gels were disulfide-dependent complexes. Note the small shift in Ero1β mobility caused by increased exposure of Ero1β thiols to the alkylating agent (Fig. 4D, compare lanes 1 and 2, lanes 3 and 4, and lanes 5 and 6). Misoxidation was reversible, since the most severe mutant, G252S, could recover after DTT washout in normal medium (Fig. 4E, lanes 3–6, *). For G252S, DTT treatment caused a reproducible increase in the proportion of higher molecular weight complexes and aggregates (Fig. 4C, lanes 5 and 6, **). This result demonstrates that Ero1 and PDI can interact, stressful environment.

Finally, we asked whether varying the temperature could also invoke behavioral changes in the mutant proteins. Thus transfected HeLa cells were incubated at 24, 37, and 42 °C for 1 h to invoke behavioral changes in the mutant proteins. Thus transfection, stressful environment.

We conclude from these experiments that both mutations in the FAD binding domain disrupt disulfide-dependent Ero1β interactions and result in misoxidation of the Ero1β protein when cells are subjected to either a reducing or temperature stress.

Implications of Ero1β Misoxidation—The human and yeast Eros have conserved Gly and His residues at the FAD binding site. Although the properties of the yeast ero1-1 and ero1-2 mutant proteins have not been examined in this study, one would predict that the equivalent mutations in Ero1p also result in stress-induced misoxidation and that the Ero1β FAD mutants will not restore viability to the ero1-1 and ero1-2 strains. We have shown that there is an inducible misoxidation defect in the equivalent Ero1β gene product upon temperature or reducing stress (Fig. 4). In the absence of FAD as a cofactor and electron acceptor, we suspect that the normal relationship between Ero’s intramolecular disulfides and its inter-molecular disulfides with PDI cannot be maintained, resulting in -SH exposure and incorrect selection of S-S bridges by the mutant Ero proteins. The fact that misoxidation could be partly reversed (Fig. 4E) may explain why oxidizing agents such as diamide can restore viability to the ero1-1 yeast strain (16, 17).

Recent work has suggested that Ero–Ero dimers contribute to Ero function (22, 32). Given that an Ero1βC396A mutant does not homodimerize efficiently, it was possible that mutations in the FAD binding domain also prevent dimerization. However, our data show that Ero–Ero associations do not depend entirely on the FAD binding domain (Fig. 3) and thus a failure to self-complex is not likely to explain the ero1-1 and ero1-2 phenotypes. Nevertheless, it remains possible that Ero–Ero intermolecular disulfide bond formation could be altered in the G252S and H254Y mutants. Structural and enzymatic studies will be required to investigate this question further.

In S. cerevisiae, the conditional ero1-1 strain is unable to oxidize PDI, and PDI remains in the reduced state in these yeast (31). Here, we show that while the equivalent G252S Ero1β mutant can bind to PDI non-covalently, intermolecular disulfide bond formation between Ero1 and PDI is largely prevented (Fig. 2). This result demonstrates that Ero1 and PDI can interact in a disulfide and flavin-fold independent manner. We show that Gly252 is required for the establishment of PDI-Ero1 disulfide bonds (Fig. 2), even though the Ero-PDI docking site and the buried FAD binding site are not likely to be in direct contact. This fits with the idea that Ero1-PDI specificity might arise from both the active site cysteines and direct protein-protein interactions (23) and that structural changes may occur during electron transfer to the CxxxC(CxxC) site in Ero1p (32).

The properties of its novel FAD fold make Ero well placed to regulate oxidative protein folding in the ER. The dual function of FAD as an electron transfer agent and as a stabilizing cofactor provides the Ero protein with the ability to catalyze oxidation reactions and, at the same time, maintain appropriate protein-protein interactions and structural stability in a rapidly changing, stressful environment.

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