Steps in reductive activation of the disulfide-generating enzyme Ero1p

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Abstract: Ero1p is the primary catalyst of disulfide bond formation in the yeast endoplasmic reticulum (ER). Ero1p contains a pair of essential disulfide bonds that participate directly in the electron transfer pathway from substrate thiol groups to oxygen. Remarkably, elimination of certain other Ero1p disulfides by mutation enhances enzyme activity. In particular, the C150A/C295A Ero1p mutant exhibits increased thiol oxidation *in vitro* and *in vivo* and interferes with redox homeostasis in yeast cells by hyperoxidizing the ER. Inhibitory disulfides of Ero1p are thus important for enzyme regulation. To visualize the differences between de-regulated and wild-type Ero1p, we determined the crystal structure of Ero1p C150A/C295A. The structure revealed local changes compared to the wild-type enzyme around the sites of mutation, but no conformational transitions within 25 Å of the active site were observed. To determine how the C150–C295 disulfide nonetheless participates in redox regulation of Ero1p, we analyzed using mass spectrometry the changes in Ero1p disulfide connectivity as a function of time after encounter with reducing substrates. We found that the C150–C295 disulfide sets a physiologically appropriate threshold for enzyme activation by guarding a key neighboring disulfide from reduction. This study illustrates the diverse and interconnected roles that disulfides can play in redox regulation of protein activity.

Keywords: disulfide bond formation; enzyme activation; flavin adenine dinucleotide; lag phase

Introduction

A fundamental question in cell biology is how a balance between thiols and disulfides is maintained in the endoplasmic reticulum (ER) to promote efficient

oxidation of proteins while preventing irreversible mispairing of disulfides.¹ The ER sulfhydryl oxidase Ero1 catalyzes formation of disulfide bonds^{2,3} and may also serve as a redox sensor, tailoring its activity according to the thiol/disulfide balance or the presence of specific reduced substrates in the compartment.^{4,5} Although Ero1 has certain structural and mechanistic features in common with other sulfhydryl oxidases,⁶ Ero1 exhibits complex kinetics not observed in other enzyme families catalyzing the same chemical reaction. This unique behavior of Ero1, which includes a pronounced lag phase observed in assays of catalytic activity on model substrates performed *in vitro*,^{4,7} may be a manifestation of the regulatory feedback mechanism to prevent over-oxidation of the ER thiol pool.⁴ Therefore, a thorough analysis of Ero1 kinetics and their biochemical and structural bases is essential for

Abbreviations: DTT, dithiothreitol; ER, endoplasmic reticulum; FAD, flavin adenine dinucleotide; PDI, protein disulfide isomerase; Pdi1p_{red}, reduced yeast PDI; PEG, polyethylene glycol; Trx, *E. coli* thioredoxin I; Trx_{red}, reduced Trx.

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Figure 1. A: Ribbon diagram of wild-type Ero1p (Protein Data Bank code: 1RP4) colored according to temperature factor, with red corresponding to high temperature factor regions and blue to low. The FAD is shown in sticks. Disulfide bonds and the unpaired C208 are illustrated in ball-and-stick representation and labeled. Eleven of the 14 cysteines in full-length yeast Ero1p are present in the truncated protein that was crystallized. B: The two-electron transfer events involved in oxidation of Pdi1p by Ero1p are illustrated as paired arrows. C: The molecular surface of Ero1p is shown from two angles with cysteine sulfur atoms colored yellow and surface-exposed hydrophobic side-chains colored green. Aromatic side chains are in a darker shade. The table lists the solvent-accessible surface areas (SAS) of the side chains of the indicated cysteines as determined using the program areaimol (CCP4 package).⁸

understanding the origin of the redox balance in the ER. A previous study presented the finding that mutation of certain cysteine residues in yeast Ero1

(Ero1p) increases enzyme activity.⁴ We now address the mechanism by which noncatalytic disulfides tune the response of the enzyme to thiol species in the environment.

Yeast Ero1p has 14 cysteine residues and a bound flavin adenine dinucleotide (FAD) cofactor [Fig. 1(A)]. Two pairs of Ero1p cysteines are on the direct electron-transfer pathway from substrate to flavin [Fig. 1(B)]. One of these pairs, a Cys-X-X-Cys motif that forms the active-site disulfide (C352–C355), abuts the isoalloxazine of the FAD and most likely transfers electrons directly to the cofactor.^{6,9} The second pair (C100–C105), the “shuttle” disulfide, is a Cys-X₄-Cys motif on a flexible loop near the active site and appears to mediate transfer of electrons from substrate proteins to the active-site disulfide.^{6,9,10} A third conserved disulfide (C90–C349) is in the vicinity of the active site but is not essential for Ero1p activity *in vitro* or under conditions that have been examined *in vivo*.⁴ Its precise role and the reason for conservation are unclear. Other disulfides, more distant from the active site, are also dispensable for activity, but they may nevertheless contribute to regulation of the enzyme. We reported that a yeast Ero1p mutant lacking the C150–C295 disulfide, which is ~35 Å from the FAD isoalloxazine, has a shortened lag phase in enzyme assays on model substrates *in vitro*, increases the intracellular ratio of oxidized to reduced glutathione, and decreases viability of yeast.⁴ These observations demonstrate an important role for Ero1p redox centers off the catalytic electron transfer pathway and distant from the active site.

A remaining question is how loss of the C150–C295 disulfide in Ero1p has such profound effects on enzyme activity and on redox homeostasis in cells, and whether the C150A/C295A mutant can provide insight into the autoregulatory mechanism of the wild-type enzyme. The structure of Ero1p, obtained previously from two crystal forms (hexagonal at 2.8 Å resolution and centered orthorhombic at 2.2 Å resolution),⁶ suggested the possibility of redox-dependent conformational changes. The temperature factor distribution [Fig. 1(A)] and a comparison of the structures from the two crystal forms highlighted the flexibility of loops in what is designated the “top” of the Ero1p structure. A congregation of disulfides in the loop-rich region and at the interface with the 10-helix core of the enzyme, together with numerous exposed hydrophobic residues and a richly featured surface containing hydrophobic pockets and grooves [Fig. 1(C)], suggests that reduction or elimination of disulfides may enable conformational rearrangement of the top portion of the enzyme, with consequent effects on enzyme activity. Conformational changes upon disulfide reduction may occur also in mammalian Ero1α, as supported by changes in intrinsic tryptophan/tyrosine fluorescence upon reduction or mutation of certain disulfides.¹¹

To test the hypothesis that elimination of the C150–C295 disulfide in Ero1p causes conformational

Figure 2. Enzyme progress curves of various Ero1p constructs. Residue number boundaries appear in subscript in the label of each curve. A: Ero1p activity was monitored by stopped-flow fluorimetry. Ero1p, FAD, and Trx_{red} were mixed under anaerobic conditions at final concentrations of 1, 100, and 50 μ M, respectively. The exogenous FAD served as the electron acceptor for Ero1p, and fluorescence decay of FAD upon reduction reported the progress of the reaction. B: Superposition of fluorescence data by rescaling Ero1₁₀₋₄₂₄ and Ero1₅₆₋₄₂₄ C150A/C295A data along the time axis provides evidence for altered regulation of Ero1₅₆₋₄₂₄ C150A/C295A activity.

changes that increase enzyme activity, we determined the structure of the C150A/C295A de-regulated Ero1p mutant by X-ray crystallography. Interpretation of this structure was facilitated by enzymatic assays of wild-type and mutant Ero1p oxidizing either native or model substrates. Tandem mass spectrometry (LC-MS/MS) analysis of Ero1p disulfide connectivity during activation and turnover revealed the basis for the hyperactivity of the C150A/C295A mutant and provided insight into the natural activation mechanism of the enzyme.

Results

Electron transfer kinetics of the C150A/C295A Ero1p mutant

Increased turnover rates and a shortened lag phase for the C150A/C295A Ero1p mutant were previously

reported based on oxygen consumption assays.⁴ To obtain greater precision at early time points, we performed similar reactions using stopped-flow fluorimetry to monitor oxidation of reduced thioredoxin (Trx_{red}) by Ero1p [Fig. 2(A)]. Free FAD was used as the electron acceptor for Ero1p instead of oxygen, since reduction of the flavin is detectable by a decrease in fluorescence. Two recombinant wild-type Ero1p constructs, spanning amino acid residues 10–424 or 56–424,^{4,6,7} were examined. These two constructs differ by approximately twofold in their activities, but their progress curves are identical in shape, as can be seen by scaling the time axes [Fig. 2(B)]. Thus, the lag phase observed during oxidation of Trx_{red} is an inherent property of the wild-type enzyme on this substrate regardless of absolute activity. The lag phase is also independent of whether molecular oxygen or FAD is used as the electron acceptor. In contrast, the C150A/C295A mutant has a progress curve qualitatively different from either wild-type version of Ero1p [Fig. 2(B)].

Ero1p C150A/C295A structure

The dramatic impact on enzyme kinetics of the C150A/C295A double mutation prompted us to investigate the structure of this Ero1p variant. We sought to reveal any conformational differences compared to wild type that may explain the differences in activity and pre-steady state kinetics. Mutant protein was produced in *E. coli* with approximately one-third the yields obtained for wild type and was isolated using a comparable protocol.⁶ As for wild-type Ero1p, crystals of Ero1p C150A/C295A were obtained in both hexagonal and centered orthorhombic forms. The orthorhombic form was chosen for further study because it diffracted to higher resolution. The Ero1p C150A/C295A structure was determined using phases calculated from a partial model of wild-type Ero1p (see Materials and Methods section). The structure was refined using diffraction data to 1.85 Å resolution (Table I).

Conformational differences compared to wild-type Ero1p were observed in the region of the missing C150–C295 disulfide (Fig. 3, left panel). These differences occur primarily in the α -helix downstream of C295 (designated helix α 6).⁶ The α 6 helix is five residues longer at the amino terminus in the C150A/C295A mutant, extending from L297 as opposed to I302, allowing D296 to cap the helix. In the wild-type structure, participation of C295 in the disulfide with C150 prevents D296 from capping the α 6 helix, and the shorter version of the helix is instead partially capped *in trans* by the side chain of S293. The two C β atoms of A150 and A295 are 9.2 Å apart in Ero1p C150A/C295A, in contrast to the 3.8 Å distance between the analogous cysteine C β atoms of the wild-type enzyme. In summary, the region in the vicinity of residues 150 and 295 apparently

Table I. Summary of Data Collection and Refinement Statistics

Ero1p mutant	C150A/C295A	C143A/C166A
Space group	C22 ₁	P6 ₂
Unit cell parameters (Å)	73.4 × 132.8 × 102.7	107.2 × 107.2 × 124.2
Resolution (Å)	500.0–1.85	50.0–3.2
Completeness (%)	99.0	99.9
Redundancy	4.9	12.4
R_{sym}^a	0.037	0.236
$\langle I/\sigma I \rangle$	15.4	5.7
Total reflections/test set	42,641/2864	13,131/672
$R_{\text{work}}/R_{\text{free}}^b$	0.209/0.238	0.244/0.293
Rms deviations from ideality		
Bonds (Å)	0.005	0.008
Angles (°)	1.314	1.847
Number of atoms		
Protein	2899	2842
Water	211	12
FAD	53	53
NEM	9	9
Cd ²⁺	1	1

^a $R_{\text{sym}} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where $I_i(hkl)$ is the observed intensity and $\langle I(hkl) \rangle$ is the average intensity for i observations.

^b $R_{\text{work}}, R_{\text{free}} = \sum \|F_{\text{obs}} - F_{\text{calc}}\| / \sum F_{\text{obs}}$, where F_{obs} and F_{calc} are the observed and calculated structure factors, respectively. A set of reflections (6.7%) were excluded from refinement and used to calculate R_{free} .

relaxes in the double cysteine-to-alanine mutant to a conformation incompatible with the disulfide but containing more regular and extensive secondary structure.

Despite these differences, most of the Ero1p C150A/C295A structure, including the active-site region (Fig. 3, right panel), is similar to that previously observed for wild-type Ero1p. The resolution of the diffraction data was significantly better than obtained for wild type, but regions that previously gave poor electron density (i.e., the 155–165 and 108–116 loops) were also apparently flexible in the Ero1p C150A/C295A crystals. No major differences were seen in the solvent exposure of other disulfide

bonds in the structure. The relatively minor differences in the C150A/C295A Ero1p structure do not rule out the possibility of global changes in protein dynamics, but any putative changes in dynamics did not prohibit crystallization. The absence of a clear structural explanation for the increased activity of the C150A/C295A mutant led us to initiate a more detailed biochemical analysis of the reductive activation process.

Gel electrophoretic analysis of disulfide reduction during Ero1p activation

Reduction of a series of disulfide bonds in Ero1p upon encounter with reducing substrate has been observed by changes in migration rate of the enzyme on denaturing gels.⁴ These experiments are performed by incubating Ero1p with substrate and blocking the reaction after various times using rapid alkylating agents to modify free thiols. For our current studies, we compared reactions blocked with the small alkylating agent *N*-ethyl maleimide (NEM) versus the larger maleimide derivative 4-acetamido-4'-maleimidylstilbene-2,2'-disulfonic acid (AMS) and analyzed the samples on both reducing and nonreducing denaturing gels. These experiments extend our previous work with AMS-modified samples resolved under nonreducing conditions⁴ and allow for additional insight into the reductive activation process of Ero1p.

The thiol trapping experiments provide information on the number of free cysteines that become reduced and the nature of any remaining disulfides in Ero1p at a given time after substrate addition. When samples are resolved under reducing conditions, the migration rate of Ero1p reflects the absolute mass of the enzyme, which depends on both the number of cysteines modified and the type of alkylating agent used. AMS contributes ~510 D per cysteine modified, whereas NEM contributes ~125 D. The small change in mass due to NEM alkylation of

Figure 3. Superposition of the structures of wild-type and C150A/C295A Ero1p. In the central panel, both structures are shown in beige. In the zoom views to either side, the Ero1p C150A/C295A structure is shown in dark red. The left zoom window is rotated relative to the central view to show structural differences with greater clarity. The dotted line indicates a region of poor electron density that could not be modeled in the mutant structure and was backbone traced in the wild-type structure. The right zoom window shows the close correspondence of the wild-type and mutant enzymes in the region of the active site.

Figure 4. Disulfide reduction during catalysis by Ero1p and the de-regulated mutant C150A/C295A. Enzyme at a final concentration of 2 μ M was mixed with 100 μ M Trx_{red}. Aliquots were removed from the reactions at the indicated time points, and disulfide status was preserved by blocking with maleimide reagents. The migration of Ero1p on SDS-PAGE at the various time points was examined and compared with wild-type Ero1p (WT), the indicated Ero1p mutants, and DTT-treated Ero1p (WT reduced). (A) The Trx_{red} oxidation reaction was blocked with AMS and separated by SDS-PAGE under nonreducing conditions. The asterisk (*) indicates a species that migrates slower than Ero1p C90A/C349A when blocked with AMS, but faster than this disulfide mutant when blocked with NEM (compare with panel C). (B) The same experiment as in (A) was performed, but the samples were reduced with DTT after AMS treatment before separation by SDS-PAGE. (C) Reactions were blocked with NEM and applied to the gel under nonreducing conditions.

free thiols does not result in an observable shift in migration of Ero1p on a reducing, denaturing gel. In contrast, under the same gel conditions, Ero1p with AMS-modified cysteines can be distinguished from unmodified Ero1p. When samples are analyzed under nonreducing conditions, the migration rate of Ero1p is influenced not only by the change in mass from modified cysteines but also by the presence or absence of disulfide bonds between cysteines distant from one another in amino acid sequence, which affects the hydrodynamic radius of the SDS-bound, denatured protein. For AMS-modified samples, the mobility of Ero1p will be influenced both by the contribution of any disulfides to the hydrodynamic radius and by the $\sim$ 510 D mass added per modified thiol. When NEM is used, the mass change due to thiol derivation is negligible, and the apparent mobility under nonreducing conditions will primarily reflect the increase in hydrodynamic radius upon

reduction of disulfides between cysteines distant from one another in amino acid sequence.

In experiments performed using Trx_{red} as a substrate, Ero1p was converted stepwise to a set of more slowly migrating species (Fig. 4), as reported previously.⁴ Appearance of these species correlates with increased enzyme activity.⁴ At later times in the reaction, when most of the substrate had been oxidized, Ero1p became re-oxidized to its initial state of low activity. For the Ero1p C150A/C295A mutant, the more slowly migrating species appeared earlier in the reaction and disappeared earlier as well (Fig. 4). Furthermore, the most slowly migrating band was never the most populated species in the Ero1p C150A/C295A reaction, whereas it was the dominant species at a certain point ($\sim$ 1 min) in the reaction with the wild-type enzyme.

As explained above, the different migration rates of Ero1p species reflect different disulfide

connectivity. A C90A/C349A mutant, which lacks the disulfide that closes the largest loop in the protein, shows a major shift in mobility relative to wild-type Ero1p; this mutant produces the largest increase in hydrodynamic radius of any single disulfide mutant relative to wild type (Fig. 4). Notably, when the reaction of wild-type Ero1p with substrate was blocked with AMS, the most slowly migrating species [asterisk in left panel of Fig. 4(A)] ran above the position of the C90A/C349A mutant. However, the same reaction blocked with NEM resulted only in species that migrated more quickly than the C90A/C349A mutant [asterisk in left panel of Fig. 4(C)]. The lack of a maximal change in the hydrodynamic radius of NEM-treated Ero1p implies that the C90–C349 disulfide may be intact under these reaction conditions. For Ero1p with an intact C90–C349 bond, the shift observed with AMS likely reflects the combined effect of the increase in mass due to thiol modification and the lesser hydrodynamic changes observed during reduction of other disulfides less distant in sequence than C90–C349. Alternatively, the slowest migrating Ero1p species could lack C90–C349, and the moderate migration shift may reflect the presence of an alternate long-range disulfide formed by thiol/disulfide exchange. A catalytic intermediate with a disulfide between C105 and C352 has been previously trapped *in vivo*,¹⁰ and the presence of this long-range disulfide could perhaps account for faster mobility even if C90–C349 were absent.

At early time points (<1 min), Ero1p blocked with NEM did not exhibit a significant shift in mobility on the gel [Fig. 4(C), left panel]. In contrast, Ero1p blocked with AMS did display species with lower electrophoretic mobility at these times on both nonreducing and reducing gels [Fig. 4(A,B)], suggesting reduction of one or more disulfides that do not greatly affect the protein hydrodynamic radius under denaturing conditions. What appeared as a single species in the NEM-blocked reaction was resolved into two distinct species by AMS treatment, which is most readily observed under nonreducing conditions but is also apparent as a closely migrating pair of bands under reducing conditions. Between 0.1 and 0.5 min, which is the time-frame corresponding to enzyme activation (Fig. 2), the ratio of the upper to lower bands of the ~40 kD doublet for the AMS-blocked reaction increased [Fig. 4(A)].

For early time points in reactions using the Ero1p C150A/C295A mutant, the single major band seen in the NEM-blocked reactions was similarly resolved into two species by blocking with AMS. However, in this case, the mutant Ero1p appeared maximally reduced at 0.1 min, and the ratio of the upper to lower bands of the ~47 kD doublet decreased between 0.1 and 0.5 min as the Ero1p mutant protein was rapidly re-oxidized. Notably, significant retardation of mobility was seen for the Ero1p

C150A/C295A mutant at the shortest time point. However, the shifted species migrate faster than fully reduced Ero1p or the C90A/C349A mutant and thus are not likely to reflect reduction of the C90–C349 disulfide.

Mass spectrometric analysis of Ero1p activation

To directly map the Ero1p disulfides that were reduced in each gel-shifted species described above, we subjected to in-gel proteolysis and tandem mass spectrometry bands from AMS-blocked reactions run under nonreducing conditions (Fig. 5). Earlier attempts at disulfide mapping of Ero1p using matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometry suggested the presence of disulfides inconsistent with the X-ray crystal structure (data not shown), revealing the risk of over-interpreting data consisting of only parent peptide masses. LC-MS/MS data, in contrast, enable more reliable assignments of peptide identities.

The first LC-MS/MS experiment [Fig. 5(B)] was performed without in-gel reduction and alkylation, such that cysteine connectivity in disulfides was preserved. The first and most rapidly migrating partially reduced species observed showed C100 in AMS-modified form and the C143–C166 and C150–C295 disulfides intact. The next shifted species showed C166 alkylated and C150–C295 in disulfide form. The most slowly migrating species showed the active-site disulfide (C352–355) to be intact and C150, C166, and C349 to be AMS-modified. Again, it should be noted that this species, when blocked with NEM rather than AMS, migrates faster than the C90A/C349A mutant [Fig. 4(C), asterisk], suggesting that it nevertheless contains a long-range disulfide. In this set of experiments, we did not observe any non-native disulfides, that is, those not present in the Ero1p crystal structure.

To complement this information, we did a second LC-MS/MS experiment, in which Ero1p species in gel slices from the same AMS-blocked experiment were reduced with dithiothreitol (DTT) and alkylated with iodoacetamide before in-gel proteolysis. This experiment revealed which cysteines were participating in disulfides (carbamidomethylated) and which were reduced (AMS-modified) at each time point [Fig. 5(C)]. Disulfide connectivity information was lost, but peptide recovery could in principle be altered or improved by reduction of the disulfide bonded peptides. This experiment was performed both on the wild-type enzyme and on Ero1p C150A/C295A. For both versions, the first Ero1p thiol to be detected in AMS-modified form was C143. The species showing reduced and modified C143 increased in intensity between 0.1 and 0.5 min for wild-type Ero1p but was present as the dominant band already at the 0.1 min time point of the Ero1p

C150A/C295A reaction. Analysis of the highest band in the wild-type reaction revealed populations of species with C150 and C349 reduced as well. The highest band of the C150A/C295A mutant also showed reduction of C349. Overall, the mass spectrometry data revealed a progressive loss of disulfides in wild-type Ero1p in the order C100—C105, C143—C166, C150—C295, and perhaps C90—C349. For the C150A/C295A mutant, the most striking and consistent observation was the appearance of C143 as an AMS-modified thiol at early timepoints.

The above mass spectrometry analyses were conducted on Ero1p after reaction with a model substrate. To determine how a native substrate affects noncatalytic disulfides of Ero1p, we performed comparable experiments using yeast Protein Disulfide Isomerase (Pdi1p). Ero1p was mixed with reduced Pdi1p (Pdi1p_{red}), and the reaction was blocked with either NEM or AMS. Because Pdi1p migrates to a similar position on electrophoretic gels as the more

reduced species of Ero1p observed in the experiments with Trx_{red}, Ero1p was visualized with a fluorescent stain specific to poly-histidine tags, and an untagged version of Pdi1p was used. Wild-type Ero1p blocked with NEM at various points in the reaction showed no species with retarded migration [Fig. 6(A), left panel], whereas blocking with AMS revealed a minor population of modified Ero1p [Fig. 6(B), left panel]. In contrast, a significant population of Ero1p C150A/C295A was shifted upon addition of Pdi1p_{red}, after blocking with either NEM or AMS [Fig. 6(A,B), right panels]. This observation suggests that a disulfide between nonvicinal cysteines is reduced in the slowly migrating fraction. Disulfide rearrangement is also a formal possibility. In these studies, no Ero1p species similar to the most slowly migrating species seen in the reactions with Trx_{red} were observed, though Ero1p and Ero1p C150A/C295A did oxidize Pdi1p_{red} under the conditions of the experiment [Fig. 6(C)]. Therefore, Ero1p is functional on Pdi1p without quantitative reduction of multiple regulatory disulfides.

After establishing that the partially reduced species of Ero1p or Ero1p C150A/C295A obtained upon reaction with Pdi1p_{red} do not comigrate with Pdi1p itself (~55 kD), we aimed to identify the disulfide that becomes reduced in the C150A/C295A mutant. We incubated Ero1p C150A/C295A for short times with Pdi1p_{red}, blocked the reaction with either

Figure 5. Mass spectrometric identification of partially reduced Ero1p species. **A:** The upper panels of Figure 4 (reaction time-courses of wild-type and C150A/C295A Ero1p blocked with AMS) are reproduced, with the bands that were subjected to mass spectrometry indicated by ovals. **B:** A summary of the mass spectrometry results from each band is indicated on a map of the Ero1p primary structure. All disulfides observed in the Ero1p crystal structures were found in the time zero band (i.e., in the absence of substrate), as indicated by the linked open circles, and no non-native disulfides were detected. The homogeneity of the time zero band, and the fact that this species was obtained from pure protein stock rather than from aliquots removed from enzymatic reactions, may explain the improved peptide coverage compared to the other bands analyzed. Reduction of C143—C166, indicated by unpaired dark circles, was seen in bands that appeared earlier than those showing reduction of C150—C295. It should also be noted that reduction of C143—C166 does not result in a mobility change in the wild-type enzyme blocked with NEM, as can be seen by comparing Figures 4(C) and 5(A). Reduction of this disulfide does, however, cause a shift when it occurs in the C150A/C295A background. **C:** The indicated bands were subject to reduction and alkylation with iodoacetamide before proteolytic cleavage and mass spectrometry analysis. Open circles indicate cysteines that were detected as modified by iodoacetamide, whereas dark circles indicate cysteines that were detected as being modified by AMS, and thus had been reduced during the reaction with Trx_{red}. Half-filled circles represent cysteines detected in both states in the same experiment.

Figure 6. The reaction between 2 μ M Ero1p or Ero1p C150A/C295A and 75 μ M Pdi1p_{red} was blocked with (A) NEM or (B) AMS after various times and subjected to SDS-PAGE under nonreducing conditions. The band indicated by an open arrowhead in the wild-type Ero1p time course is a contaminant from the Pdi1p preparation, as it appears also in the Pdi1p control lane. A significant fraction of Ero1p C150A/C295A shows retarded mobility at early time points when blocked with either NEM or AMS (asterisks). A band corresponding to that marked with an asterisk in NEM-blocked Ero1p C150A/C295A was analyzed by LC-MS/MS, and the C143–C166 disulfide was found to be reduced. (C) Oxidation of Pdi1p by Ero1p and Ero1p C150A/C295A under similar conditions as the experiments in (A) and (B). Pdi1p_{red} was mixed with wild-type or mutant Ero1p. Aliquots were removed at various times and reacted with PEG-maleimide of 5 kD. Mal-PEG modified Pdi1p species are indicated in the top portion of the gel. The band labeled Pdi1p_{ox} has both active sites oxidized and was thus resistant to PEGylation.

NEM or AMS, separated the proteins by SDS-PAGE, and stained with Coomassie (gel not shown). In the AMS-treated sample, the higher band derived from the Ero1p mutant comigrated with a Pdi1p contaminant or degradation product and was not analyzed. In contrast, in the NEM-treated sample, the band apparently corresponding to that labeled with an asterisk in the right panel of Figure 6(A) could be identified and excised. Tandem mass spectrometry analysis of this band showed both C143 and C166 in NEM-modified form, demonstrating that C143–C166 had been reduced by Pdi1p_{red}. A single six-amino acid peptide containing reduced and modified C90 was also observed, although the species analyzed migrated much more quickly in the gel than the C90A/C349A mutant and is thus likely to contain a long-range disulfide. Displacement of C90 by attack of C143, or more likely C166 (see below), on

C349 might occur in a fraction of the Ero1p molecules, yielding a species with similar hydrodynamic radius to that having C143 and C166 reduced and the remaining disulfides intact.

Ero1p C143A/C166A structure

The identification of C143–C166 as the disulfide that is reduced much more rapidly in the hyperactive C150A/C295A Ero1p mutant prompted us to investigate the structural changes that occur in Ero1p upon removal of this disulfide. The Ero1p mutant C143A/C166A was produced in *E. coli* with ~20% the yields obtained for wild type. Crystals were obtained in the primitive hexagonal form with unit cell dimensions similar to those of crystals obtained with wild-type Ero1p in this space group (Table I). The Ero1p C143A/C166A structure was refined using diffraction data to 3.2 Å resolution.

Figure 7. C143 and C166 play different roles in the Ero1p structure. A: Stereo image of a superposition of the structures of wild-type (colored beige) and C143A/C166A Ero1p (dark red). Disulfides and the carboxy terminus are labeled. The endpoints of a large missing loop are indicated by circles and labeled with the number of the last modeled residue in each case. Mutation of C143 and C166 increases the length of the disordered loop such that an additional ~ 10 residues cannot be modeled. To emphasize the missing loop, the view is different from in Figure 3, but it corresponds to the summary Figure 8. B: A close-up view of the region around C143 shows the local structural differences between the wild-type Ero1p structure and the C143A/C166A mutant. The C β atom of the alanine at position 143 in the mutant is shown in blue and labeled. Side chains of three acidic residues immediately upstream of C143 are shown as blue and red sticks. To illustrate the extent of the conformational differences in this area, the side chain of D140 is labeled in both the mutant and wild-type structures. C: A 2Fo-Fc electron density map, contoured at 1σ , illustrates the lack of interpretable density corresponding to the loop containing the C166A mutation. D: Activities of single mutants disrupting the C143–C166 disulfide obtained from a gel-based Trx oxidation assay. Oxidation of Trx_{red} by the indicated Ero1p variants was blocked at various time points by addition of PEG-maleimide 5 kD. Oxidized Trx is resistant to PEG modification and thus migrates faster than modified Trx in SDS-PAGE. The band intensities of oxidized and reduced/modified Trx were quantified and plotted as fraction reduced. The C143A mutant, which retains C166, is hyperactive, but the C166A mutant, retaining C143, is not. This finding points to a role for the C166 thiol in Ero1p activation.

As for the C150A/C295A mutant, local rearrangements of the polypeptide chain were seen in the C143A/C166A around the mutation site. However, in contrast to the C150A/C295A mutant, in which both polypeptide segments containing the cysteine-to-alanine mutations acquired more regular secondary structure upon mutation, the C143A/C166A double mutant is more disordered than wild type [Fig. 7(A)]. A cluster of acidic residues (D137, D138, D140, D141, and E142) immediately upstream of C143A assumes an alternate conformation [Fig. 7(B)], without an increase in secondary structure. The region surrounding C166A loses its connection to the helical core of the enzyme, and the polypeptide chain between residues 154 and 175 cannot be traced in electron density maps due to poor electron density [Fig. 7(C)]. In the wild-type Ero1p structures and in C150A/C295A, electron density corresponding to the region around residues 155–165 was also poor or uninterpretable. However, the

disordered region is significantly extended, in the direction of the active site, in the C143A/C166A mutant.

Kinetics studies of single and double mutants of the C143–C166 disulfide

The structural changes seen in the Ero1p C143A/C166A mutant are not sufficient to activate the enzyme; this mutant, when examined previously, was found not to be hyperactive but rather slightly slower than wild-type Ero1p.⁴ To determine whether the thiol form of either cysteine participating in this bond may have a direct role in activation, we constructed the single cysteine-to-alanine mutants C143A and C166A. The C166A mutant behaved similarly to the C143A/C166A double mutant. However, the rate of oxidation of Trx_{red} by the C143A mutant was found to be indistinguishable from the rate of oxidation by the de-regulated C150A/C295A mutant in a gel-based assay [Fig. 7(D)]. This observation

suggests that liberating C166 is an important step in activating Ero1p. However, the mutagenesis experiment did not rule out the possibility that the C143A mutant is hyperactive for the trivial reason that the unpaired C166 disrupts the C150–C295 disulfide and produces a species that mimics C150A/C295A. Indeed, mass spectrometry analysis of the C143A mutant showed some C295 in reduced and alkylated form, suggesting that C166 displaces it and forms a disulfide with C150 in a fraction of the molecules. It should be noted that this species was not observed in any experiment in which C143–C166 became reduced during an enzymatic reaction, suggesting that it is an artifact of lengthy exposure of C166 during enzyme preparation and purification. The activity of C143A Ero1p on Pdi1p_{red} was greater than that of wild-type Ero1p but not as rapid as C150A/C295A (data not shown). This observation may indicate that a non-native disulfide bond formed when C143 is missing is reduced less effectively by Pdi1p_{red} than by Trx_{red}. Together, the study of single-cysteine mutants C143A and C166A supports the conclusion based on crystallographic studies that the C166 region has a large range of motion when freed from C143, and suggests a specific role for C166.

Discussion

The mechanism by which Ero1p activity is controlled by encounter with reducing substrates is directly related to the ability of the enzyme to maintain redox balance in the ER. The various Ero1p disulfides have distinct roles in catalysis and control of enzyme activity, and the experiments presented here were designed to dissect these roles. The prior observation that eliminating the C150–C295 disulfide of yeast Ero1p increases enzyme activity, coupled with the observation of cascaded reduction of Ero1p disulfides in gel assays using Trx_{red} as a substrate, suggested that reduction of C150–C295 is part of the series of events that results in activation of the enzyme.⁴ The reduction of C150–C295 was considered as a possible early event in the cascade, whereas the longest range disulfide (i.e., C90–C349) was surmised to open later in the reaction.⁴ One puzzle in this model was the implication that the C150–C295 disulfide should be reduced rapidly in the C90A/C349A Ero1p mutant to yield a fully activated enzyme. The C90A/C349A mutant would thus be expected to have a short lag phase and be hyperactive, but instead it has only a slightly shortened lag phase and essentially wild-type activity.

The LC-MS/MS results presented here consistently showed reduction of the C143–C166 disulfide preceding reduction of other noncatalytic disulfides. The opening of C143–C166 first in the cascade was unexpected, considering the lack of solvent exposure of this disulfide in the Ero1p crystal structure⁶ and

the observation that the C143A/C166A double mutation did not activate Ero1p.⁴ The disulfide mapping further indicated that reduction of C150–C295 is actually a late step in Ero1p activity assays on Trx_{red}. This result implies that the increased activity of the C150A/C295A mutant cannot simply be ascribed to removal of an initial hurdle in the reductive activation process. Whether reduction of the C150–C295 disulfide is required as the second step in the activation of Ero1p is difficult to test using Trx_{red} as a substrate, since reduction of the C150–C295 disulfide inevitably followed reduction of C143–C166 temporally (Fig. 5). In contrast to the relative ease of eliminating a disulfide bond by mutagenesis, specifically stabilizing a disulfide to test the effect of a lack of its reduction presents a major experimental challenge.

Remarkably, the observations presented here with the native substrate Pdi1p_{red} suggest that reduction of multiple Ero1p disulfides may not be required before Ero1p can oxidize substrate. When Pdi1p_{red} was mixed with wild-type Ero1p, no shifted bands corresponding to reduction of C150–C295 (or the longer range disulfide C90–C349) were observed at any point in the reaction [Fig. 6(A), left panel], although wild-type Ero1p does oxidize at least one of the domains of Pdi1p_{red} in this time frame [Fig. 6(C), left panel]. Stoichiometric reduction of C150–C295 is thus not an obligatory step in generating active Ero1p. It is possible, however, that Pdi1p_{red} reduces multiple disulfides in an undetectable subpopulation of Ero1p, and this fraction is then extremely active on Pdi1p_{red} and performs all the substrate oxidation observed, while the vast majority of Ero1p molecules remain oxidized and inactive. We find this explanation unlikely. If Pdi1p_{red} were inefficient at activating Ero1p but then served as an excellent substrate of the activated fraction, lag phase kinetics would be observed for oxidation of Pdi1p_{red}. In fact, the lag phase is more pronounced during oxidation of Trx_{red} than of Pdi1p_{red}.

Since reduction of C150–C295 does not seem to be required for Ero1p turnover, we are left with the question of why an enzyme variant lacking this disulfide shows dramatically increased activity, on both model and native substrates.^{4,12} The major finding presented here is that the C150–C295 disulfide affects the reactivity of the neighboring disulfide, between C143 and C166, and that opening of the latter correlates with exit from the lag phase and increased oxidase activity. The non-native substrate Trx_{red} reduces C143–C166 in wild-type Ero1p apparently to completeness, whereas the native substrate Pdi1p_{red} is inefficient at reducing this disulfide or at maintaining it reduced as Ero1p competes to re-oxidize it. When C150–C295 is eliminated by mutation, the C143–C166 disulfide is reduced more rapidly by Trx_{red} and more extensively by Pdi1p_{red}. The greater

portion of the enzyme with the C143–C166 disulfide reduced could explain the greater activity of the C150A/C295A mutant compared to wild-type Ero1p in the oxidation of Pdi1p_{red}.

Differences in substrate oxidation kinetics have also been observed for cysteine mutants in recombinant human Ero1 α . In particular, mutation of Ero1 α C131 increased slightly the rate of oxidation of Pdi1p *in vitro*¹¹ and increased cellular GSSG levels.⁵ The explanation for increased activity of the human Ero1 α C131A mutant is distinct from the mechanism outlined herein for Ero1p C150A/C295A. Ero1 α C131 was proposed to form an inhibitory disulfide with Ero1 α C94,¹¹ which is one of the cysteines participating in the shuttle disulfide of the mammalian enzyme. The increased activity of the Ero1 α C131A mutant was proposed to reflect loss of the inhibitory C94–C131 disulfide and ability of the shuttle disulfide between C94 and C99 to form properly. Although yeast Ero1p does not oxidize Trx_{red} significantly at time zero of the reaction, an inhibitory disulfide formed between a regulatory and a shuttle cysteine cannot explain this inhibition; an intact shuttle disulfide was observed in the crystal structure of yeast Ero1p and by disulfide mapping using LC-MS/MS [Fig. 5(B) and Supporting information]. If a non-native disulfide were to be present during reactions of yeast Ero1p, it would most likely be activating rather than inhibitory. However, numerous mass spectrometry samples of Ero1p trapped during reactions with Trx_{red} have failed to reveal non-native disulfides, despite high peptide coverage of the protein sequence and excellent recovery of native disulfides. If such species exist, they may be transient or poorly populated.

If not by engaging shuttle cysteines, then how might Ero1p regulatory disulfides restrain enzyme activity? For both C143–C166 and C150–C295, the disulfide constrains the polypeptide to conformations different from those observed when the disulfides are removed. In particular, we observed that the entire loop between residues 155 and 175 becomes disordered when C166 is not tethered to C143, as shown in the structure of the Ero1p C143A/C166A mutant. The dramatically extended range of motion of this loop may allow it to impact the active site. As the C143A/C166A double mutant is not hyperactive, however, C166 may be specifically required for these effects. What changes in the active-site region might increase the rate of catalysis? One hypothesis is that the active-site disulfide (C352–C355) of Ero1p is poorly accessible for redox communication with the shuttle disulfide (C100–C105). In the structure of another yeast sulfhydryl oxidase, Erv2p,¹³ the shuttle disulfide was observed within dithiol-disulfide exchange distance of the active-site disulfide. In contrast, the active-site disulfide of Ero1p is less approachable.⁶ Although the shuttle disulfide is

Figure 8. Summary of roles for regulatory disulfides distant from the Ero1p active site. Reduction of the C150–C295 disulfide is not an early step in Ero1p activation. Instead, the presence of this disulfide appears to protect the neighboring C143–C166 disulfide from reduction. Reduction of C143–C166 may be a key event in Ero1p activation, allowing the polypeptide chain in the vicinity of C166 in particular to sample new conformations (the arrow indicates schematically putative motion in the direction of the active site). Though C143–C166 is nearly 25 Å from the active-site (C352–C355) disulfide in the ground-state Ero1p structure, a liberated C166 would be present on a segment of polypeptide that may be loosely tethered enough to bridge this distance.

closer to the active site in one of the Ero1p crystal forms than in the other, it is still not in direct contact and is separated from the active site by an intervening Tyr side chain (Tyr 191). The low solvent accessibility of the active-site disulfide in Ero1p is not altered by *in silico* removal of the shuttle disulfide loop (data not shown), suggesting that it is buried by other parts of the structure, occluding it from solvent as well as from the shuttle disulfide. Conformational or dynamic changes, propagating from reduction of regulatory disulfides, may facilitate redox communication between the solvent accessible shuttle disulfide and the buried active-site disulfide, forming a complete redox path from substrate to FAD.

In conclusion, we determined that the C143–C166 disulfide is the first regulatory disulfide to be reduced during Ero1p activation and that the facile activation of the C150A/C295A mutant can be explained by its increased susceptibility to reduction of C143–C166 (Fig. 8). The presence or absence of the C150–C295 disulfide 35 Å away from the active site thus affects the fate of the key C143–C166 disulfide 25 Å from the active site, and elimination of the C143–C166 disulfide allows conformational changes that may finally propagate to the active site itself, with a consequent increase in thiol oxidase activity.

Materials and Methods

Enzyme and substrate preparation

Ero1p, Ero1p C143A, Ero1p C166A, Ero1p C150A/C295A, and Ero1p C143A/C166A, all spanning

residues amino acid 56–424 of the yeast protein, were expressed in the Origami B(DE3) plysS *E. coli* strain (Novagen) downstream of glutathione *S*-transferase (GST) and an internal His₆ tag using a modified pGEX-4T1 plasmid (Amersham).⁶ Wild-type Ero1p spanning residues 10–424 was produced from a pGEX-4T1 vector without the additional His₆ tag. Bacteria were grown at 37°C to an optical density of 0.6 at 600 nm, at which point isopropyl-1-thio- β -D-galactopyranoside was added to a final concentration of 0.5 mM to induce protein expression. The growth temperature was shifted to 25°C, and cells were harvested 12–16 h later. After cell lysis, proteins were purified using Ni-NTA agarose (for constructs containing His₆ tags), cleaved with thrombin to remove the GST, and re-purified over Ni-NTA. The longer Ero1p construct, lacking the His₆ tag, was purified using glutathione sepharose beads, and thrombin cleavage was performed on the column to release Ero1p. The enzymes were then concentrated and run on a HiLoad 16/60 Superdex 75 prep grade size exclusion column monitored at 280 and 450 nm. The peaks corresponding to monomeric protein were collected. Enzymes used for crystallization were dialyzed against 10 mM Tris, 25 mM NaCl, pH 8 and concentrated to ~400 μ M (~18 mg/mL).

Trx was expressed, purified, and reduced as previously described,⁶ except that Triton X-100 was not used. For experiments in which gels were stained with Invision His₆-tag stain, Pdi1p was produced in BL21(DE3) plysS *E. coli* cells as a fusion protein with GST using the pGEX-4T1 plasmid, purified over glutathione-sepharose beads, cleaved with thrombin, and re-applied to glutathione sepharose to remove the GST. For other experiments, Pdi1p was produced with a carboxy-terminal His₆-tag as described.¹⁴

For oxidation assays, Trx was reduced with 100 mM DTT for 1 h and then desalted using a PD-10 column (GE Healthcare) equilibrated in 50 mM phosphate buffer, pH 7.5, 65 mM NaCl, 0.5 mM EDTA. Pdi1p was reduced by incubating with 10 mM GSH from a stock titrated to pH 7.0. Proteins were then desalted on a PD-10 column equilibrated in 50 mM phosphate buffer, pH 7.5, 300 mM NaCl, 0.5 mM EDTA. The concentration of reduced protein thiols was determined using Ellman's assay.¹⁵

Crystallization and structure determination

Crystals of Ero1p C150A/C295A were grown by hanging drop vapor diffusion at 20°C in 100 mM cacodylic acid pH 6–6.5, 9–15 mM cadmium sulfate, 2% methanol, 2% ethanol, and 0.8–1.6M sodium acetate. These crystals were of space group C222₁. Crystals of Ero1p C143A/C166A were grown by hanging drop vapor diffusion at 20°C in 100 mM cacodylic acid pH 6–6.5, 12–14 mM cadmium sulfate, 2% methanol,

2% ethanol, and 1.6M sodium acetate. These crystals were of space group P6₂. Before flash freezing, crystals were soaked in mother liquor with 15% ethylene glycol for a few minutes and then transferred to a 1:1 mixture of mineral oil and paratone oil (Exxon). Diffraction data for Ero1p C150A/C295A were collected at the European Synchrotron Radiation Facility beamline ID14-3. Data for Ero1p C143A/C166A were collected using a RU-H3R generator (Rigaku) equipped with an Raxis IV++ image plate system and osmic mirrors. Data were processed using HKL2000.¹⁶ Phases were calculated for the C150A/C295A mutant using the wild-type Ero1p structure after removal of residues 146–166 and 291–302, spanning the mutated cysteines. Phases were calculated for the C143A/C166A mutant after removal of residues 91–121 and 136–175. Rigid body refinement was performed before generating electron density maps. The Ero1p C150A/C295A and Ero1p C143A/C166A models were rebuilt in O¹⁷ and Coot,¹⁸ respectively, and refined using CNS.¹⁹ The quality of the final models was verified using MolProbity.²⁰

Stopped-flow fluorescence

Studies were conducted at 25°C on an Applied Photophysics stopped-flow apparatus fitted with an anaerobic adaptor. Exogenous FAD (100 μ M) rather than oxygen was used as a terminal electron acceptor, since changes in fluorescence can be monitored as FAD is reduced to FADH₂. Excitation was at a wavelength of 450 nm, and an emission cut-off filter of 495 nm was used. Enzyme and substrate preparations were made anaerobic by N₂ bubbling and pipetting in N₂ atmosphere in a glove box. To insure anaerobic conditions, solutions were supplemented with 0.1% w/v glucose and trace amounts of glucose oxidase and catalase. Samples were transferred from the anaerobic chamber in gas-tight syringes (Hamilton). Equal volumes of enzyme and Trx_{red} (prepared as described above) in 50 mM phosphate buffer, pH 7.5, 65 mM NaCl, and 1 mM EDTA were injected into the mixing/detection chamber to achieve final concentrations of 1 μ M enzyme and 50 μ M substrate.

Gel-based oxidation assays

Oxidation reactions used to analyze Ero1p electrophoretic mobility were performed at an enzyme (wild-type or mutant) concentration of 2 μ M. Trx_{red} was present at a final concentration of 100 μ M (fully reduced protein), and Pdi1p_{red} at a concentration of 300 μ M thiols (≥ 75 μ M protein). Samples from the reactions were taken at different time points and quenched by adding 1:4 v/v sample buffer (125 mM Tris, pH 6.8, 5% SDS, 50% glycerol, 0.1% bromophenol blue) containing 5 mM AMS (Molecular Probes), 100 mM NEM, or ~1 mM PEG-maleimide of 5 kD (Nektar Therapeutics). These samples were then run

on 15% denaturing polyacrylamide gels [Figs. 4 and 5(A)], or 12% gels for the kinetics of Pdi1p_{red} oxidation [Fig. 6(C)], which were then stained with Coomassie. Gels used to visualize Ero1p or Ero1p C150A/C295A in reactions with Pdi1p_{red} were stained with InVision™ His-tag In-gel Stain (Invitrogen) and scanned using a FLA-5100 fluorescent image analyzer (FUJI) with a 532 nm LPG filter. Reduced samples [Fig. 4(B)] were run with an excess of DTT.

Rates of oxidation of Trx_{red} by various mutants of Ero1p [Fig. 7(D)] were quantified using 0.5 μM enzyme and 100 μM Trx_{red}. At each time point, 10 μL were removed and mixed with 10 μL 5 mM PEG-maleimide 5 kDa in 2% SDS, 50 mM Tris, pH 6.8, 0.1% bromophenol blue, 20% glycerol. Samples were applied to a 15% denaturing polyacrylamide gel, and Trx_{red} and Trx_{ox} were visualized after separation with Coomassie stain. Band intensities were determined using the ImageQuant 5.0 program.

In-gel digestion

Protein bands were excised from SDS gels that had been stained with Coomassie and destained using multiple washings with 40% methanol and 10% acetic acid. The protein bands were either reduced, alkylated, and in-gel digested as described,²¹ or else digested directly without reduction and alkylation. Digestions were performed in 50 mM ammonium bicarbonate at 37°C using various combinations of the following sequencing grade proteases (Roche Diagnostics): bovine trypsin, bovine chymotrypsin, and *Pseudomonas* asp-N, all at a concentration of 12.5 ng/μL. Peptide mixtures were extracted from the gels with 80% CH₃CN, 1% CF₃COOH, and the organic solvent was evaporated in a vacuum centrifuge. The resulting peptide mixtures were reconstituted in 80% formic acid and immediately diluted 1:10 with Milli-Q water before mass spectrometry analysis.

Mass spectrometry

Samples were analyzed in an LTQ Orbitrap (Thermo Fisher Scientific) operated in the positive ion mode and equipped with a nanoelectrospray ion source. Peptide mixtures were separated by online reversed-phase nanoscale capillary LC and analyzed by tandem mass spectrometry (LC-MS/MS). For the LC-MS/MS, samples were injected onto a 15 cm reversed phase spraying fused-silica capillary column (inner diameter 75 μm) made in-house and packed with 3 μm ReproSil-Pur C₁₈AQ media (Dr. Maisch GmbH, Ammerbuch-Entringen, Germany) using an UltiMate 3000 Capillary/Nano LC System (LC Packings, Dionex). The LC setup was connected to the Orbitrap. The flow rate through the column was 250 nL/min, and the injection volume was 5 μL. Peptides were separated with a 50 min gradient from 5 to 65% ace-

tonitrile (buffer A: 5% acetonitrile, 0.1% formic acid, 0.005% TFA; buffer B: 90% acetonitrile, 0.2% formic acid, 0.005% TFA). The voltage applied to the union in order to produce an electrospray was 1.2 kV. The mass spectrometer was operated in the data-dependent mode. Survey MS scans were acquired in the Orbitrap with the resolution set to a value of 60,000. Up to the six most intense ions per scan were fragmented and analyzed in the linear trap. For the analysis of peptides, survey scans were recorded in the FT-mode followed by data-dependent collision-induced dissociation (CID) of the six most intense ions in the linear ion trap (LTQ). Raw spectra were processed using open-source software DTASuperCharge (<http://msquant.sourceforge.net>). The data were searched with MASCOT (Matrix Science, London, UK) against a Swiss-prot database with manually incorporated Ero1p or mutant protein (Ero1p C150A/C295A). Search parameters included variable modifications of 57.02146 Da (carboxyamidomethylation) on Cys, 510.04028 Da (AMS; hydrolyzed) on Cys, 15.99491 Da (oxidation) in Met and 0.984016 Da (deamidation) on Asn and Gln. The search parameters were as follows: maximum two missed cleavages, initial precursor ion mass tolerance 10 ppm, fragment ion mass tolerance 0.6 Da. The identity of the peptides were concluded from the detected CID products by Mascot and Sequest software and confirmed by manual inspection of the fragmentation series. The program MassMatrix was used for disulfide identification.²²

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