Human ER Oxidoreductin-1α (Ero1α) Undergoes Dual Regulation through Complementary Redox Interactions with Protein-Disulfide Isomerase

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In the mammalian endoplasmic reticulum, oxidoreductin-1α (Ero1α) generates protein disulfide bonds and transfers them specifically to canonical protein-disulfide isomerase (PDI) to sustain oxidative protein folding. This oxidative process is coupled to the reduction of O2 to H2O2 on the bound flavin adenine dinucleotide cofactor. Because excessive thiol oxidation and H2O2 generation cause cell death, Ero1α activity must be properly regulated. In addition to the four catalytic cysteines (Cys94, Cys99, Cys104, and Cys131) that are located in the flexible active site region, the Cys208–Cys241 pair located at the base of another flexible loop is necessary for Ero1α regulation, although the mechanistic basis is not fully understood. The present study revealed that the Cys208–Cys241 disulfide was reduced by PDI and other PDI family members during PDI oxidation. Differential scanning calorimetry and small angle X-ray scattering showed that mutation of Cys208 and Cys241 did not grossly affect the thermal stability or overall shape of Ero1α, suggesting that redox regulation of this cysteine pair serves a functional role. Moreover, the flexible loop flanked by Cys208 and Cys241 provides a platform for functional interaction with PDI, which in turn enhances the oxidative activity of Ero1α through reduction of the Cys208–Cys241 disulfide. We propose a mechanism of dual Ero1α regulation by dynamic redox interactions between PDI and the two Ero1α flexible loops that harbor the regulatory cysteines.

Many secretory and membrane proteins are synthesized in the endoplasmic reticulum (ER),2 in which proteins acquire their three-dimensional structures frequently through disulfide bond formation (1, 2). In the mammalian ER, the network for the catalysis of protein disulfide bond formation (oxidative protein folding) comprises a number of oxidoreductases that include more than 20 protein-disulfide isomerase (PDI) family members and five or more PDI-oxidizing enzymes such as ER oxidoreductin-1α (Ero1α) (3–6). Most PDIs contain the CysXaa-Xaa-Cys motif within the redox active sites of their thioredoxin (Trx)-like domains that catalyze disulfide introduction, isomerization, or reduction in substrate proteins. Our previous biochemical studies indicated that ERp46 and P5, in concert with the preferred partner peroxiredoxin 4 (7, 8), are dedicated to the rapid but promiscuous introduction of disulfides during the early stages of oxidative protein folding (5). Conversely, PDI, in combination with Ero1α, is involved in the later folding steps that involve selective formation of native disulfide bonds and/or the proofreading of non-native disulfide bonds (5). Other PDI-oxidizing enzymes such as glutathione peroxidases 7 or 8 (GPx7/GPx8) (9) and vitamin K epoxide reductase are also reported to potentially contribute to oxidative protein folding (10).

2 The abbreviations used are: ER, endoplasmic reticulum; Ero1α, ER oxidoreductin-1α; PDI, protein-disulfide isomerase; GPx7/8, glutathione peroxidase isozyme 7/8; AMS, 4-acetamide-4’-maleimidylstilbene-2,2’-disulfonic acid; CBB, Coomassie Brilliant Blue; GR, glutathione reductase; MBP, maltose binding protein; Ero1α-AA, Ero1α-C104A/C131A double mutant; Ero1α-AASS, Ero1α-C104A/C131A and C208S/C241S quadruple mutant; Ero1α-Cysless, Ero1α-C94A/C99A/C104A/C131A quadruple mutant; Ero1α-Cysless-SS, Ero1α-C94A/C99A/C104A/C131A and C208S/C241S sextuple mutant; Ero1α-WTSS, Ero1α-C208S/C241S double mutant; PO, partially oxidized; DSC, differential scanning calorimetry; SAXS, small angle X-ray scattering; EOM, ensemble optimization method; FAD, flavin adenine dinucleotide.
Ero1 family members are highly conserved in eukaryotes, and two isoforms, Ero1α and Ero1β, are found in vertebrates. Ero1α is expressed ubiquitously (11), whereas Ero1β is mostly expressed in the pancreas and stomach (12, 13). Although yeast Ero1p is essential for cell viability (14), Ero1α/β double-knockout mice do not display a significant phenotype, and oxidative folding of immunoglobulin proceeds normally in double knock-out cells (15). These results indicate the presence of backup systems that can complement and compensate for Ero1 in mammals.

When Ero1α oxidizes PDI, flavin adenine dinucleotide (FAD) bound to Ero1α accepts electrons from PDI and subsequently reduces O₂ to H₂O₂ (4), which is further reduced to H₂O by Ero1α-associated GPx7/GPx8 (9, 16, 17). However, excessive formation of disulfide bonds and H₂O₂ can cause cell death (18), and hence it is essential that Ero1α activity is strictly regulated. The disulfide bond pattern among four regulatory cysteines (Cys⁹⁴, Cys⁹⁹, Cys¹⁰⁴, and Cys¹³¹) in the so-called electron shuttle loop (residues Asp⁹⁰–Cys¹³¹) is a primary determinant of Ero1α activity; the catalytic Cys⁹⁴–Cys⁹⁹ active site disulfide is present in the active form, whereas Cys⁹⁴–Cys¹³¹ and Cys⁹⁹–Cys¹⁰⁴ disulfides are present in the inactive form (19–22). More recently, we demonstrated that in addition to these four regulatory cysteines, the Cys²⁰⁸–Cys²⁴¹ pair that is conserved in vertebral Ero1 family enzymes has an auxiliary role in the regulation of Ero1α. The Ero1α C104A/C131A/C208S/C241S quadruple mutant displays higher PDI oxidation activity and H₂O₂ production and is therefore more toxic than the Ero1α C104A/C131A double mutant (18). Furthermore, under high turnover conditions in which reduced PDI is constantly regenerated by reducing reagents such as GSH, the presence of Cys²⁰⁸ and Cys²⁴¹ was required for maximal PDI oxidation activity (23). Additionally, formation of the Ero1α-PDI complex involves Cys²⁰⁸ and/or Cys²⁴¹ of Ero1α in human cells (18), and the Cys²⁰⁸–Cys²⁴¹ pair interacts with PDI to fine-tune Ero1α activity.

Importantly, all of the aforementioned regulatory cysteines are located in flexible loop regions of Ero1α. It is assumed that the redox state of these cysteines influences the conformational dynamics of the flexible loops, thereby altering Ero1α activity. Consistent with this, the electron shuttle loop (loop I) that includes the Cys⁹⁴–Cys⁹⁹ disulfide in the active form is more flexible and has a higher affinity for PDI than the loop containing Cys⁹⁴–Cys¹³¹ and Cys⁹⁹–Cys¹⁰⁴ disulfides in the inactive form, which facilitates electron transfer from PDI to the bound FAD cofactor (21). In the present study, we focused on the role of another flexible loop flanked by Cys²⁰⁸ and Cys²⁴¹ (loop II) in the regulation of Ero1α activity (see Fig. 1A). The results showed that the Cys²⁰⁸–Cys²⁴¹ disulfide was significantly reduced during Ero1α-catalyzed oxidation of PDI, resulting in a more active Ero1α. Deletion of loop II prevented the reduction of the Cys²⁰⁸–Cys²⁴¹ disulfide by PDI, but the overall structure of Ero1α was not altered. Loop II therefore serves as a platform for functional interplay with PDI, which in turn elevates Ero1α activity through the reduction of the Cys²⁰⁸–Cys²⁴¹ disulfide. The present findings shed new light on the mechanisms of PDI-mediated Ero1α regulation in the mammalian ER.

**Results**

**Reduction of the Cys²⁰⁸–Cys²⁴¹ Disulfide during Ero1α-catalyzed PDI Oxidation**—Our previous observation that the Ero1α C104A/C131A/C208S/C241S quadruple mutant oxidizes PDI more efficiently and produces more H₂O₂ than the Ero1α C104A/C131A double mutant suggests that the Cys²⁰⁸–Cys²⁴¹ disulfide has a role in regulating Ero1α (18, 23). To investigate the reduction of the Cys²⁰⁸–Cys²⁴¹ disulfide by PDI during Ero1α catalysis, which leads to further elevation of Ero1α activity, we first sought to measure the change in redox state of Ero1α during PDI oxidation. However, because of significant overlap of these two similar-sized proteins in SDS-PAGE, it was difficult to monitor PDI-induced redox state changes of Ero1α using the method (data not shown). We therefore fused maltose binding protein (MBP) to the N terminus of PDI and reacted the resulting fusion protein (MBP-PDI) with Ero1α.

Ero1α in combination with MBP-PDI consumed molecular oxygen at almost the same rate as when in combination with PDI (Fig. 1B). As previously observed with PDI (18), MBP-PDI was oxidized more efficiently by Ero1α-AASS (C104A/C131A and C208S/C241S quadruple mutant) than by Ero1α-AA (C104A/C131A double mutant) (Fig. 1C, upper left panel). Thus, the fusion of MBP had a minimal effect on the reaction between PDI and Ero1α. A weak but significant band (marked by red) did appear in the upper part of the Ero1α-AA band (marked by oxi) during the early stages (0.25–1.5 min) of MBP-PDI oxidation by Ero1α-AA and disappeared as the reaction proceeded to completion (Fig. 1C, upper left panel). This band marked red was not observed with Ero1α-AASS in which Cys²⁰⁸ and Cys²⁴¹ were mutated to Ser (Fig. 1C, upper right panel). These results suggested that a portion of Ero1α underwent reduction of the Cys²⁰⁸–Cys²⁴¹ disulfide by reaction with reduced PDI. Ero1α-Cysless (C94A/C99A/C104A/C131A quadruple mutant), which is incapable of oxidizing PDI via its active site, generated an even larger amount of the red species throughout the entire reaction (0.25–10 min; Fig. 1C, middle left panel). As expected, the red band was hardly detectable for Ero1α-CyslessSS (C94A/C99A/C104A/C131A and C208S/C241S sestuple mutant; middle right), as was the case for Ero1α-AASS. The slight appearance of this band with the Ero1α-CyslessSS mutant during the later stages of the reaction may indicate only partial reduction of the two solvent-exposed disulfide bonds in the N-terminal region (Cys³⁵–Cys⁴⁸ and Cys³⁷–Cys⁴⁹). It is conceivable that the Cys²⁰⁸–Cys²⁴¹ disulfide in Ero1α could be reduced to a significant extent by PDI during catalysis provided that sufficient amounts of reduced PDI are present.

The red species was also abundant with Ero1α-WT (Fig. 1C, lower left panel), and despite the C208S/C241S double mutations, this was also the case with Ero1α-WTSS, albeit to a lesser extent than with Ero1α-WT (Fig. 1C, lower right panel). This observation suggests that a disulfide bond, possibly in the loop I of Ero1α, can also be reduced (to some extent) by PDI.

To confirm that the red band actually corresponded to a species with a reduced Cys²⁰⁸ and Cys²⁴¹, we carried out peptide footprinting analysis. Gel fragments corresponding to the red and oxi bands of Ero1α-Cysless were individually subjected to
in-gel digestion, and the resulting short peptides were separated by HPLC and analyzed by MALDI-TOF MS (Fig. 1, D and E). The peptide fragments identified are listed in supplemental Table S1. The elution peak marked by # had a molecular mass equivalent to that of Ero1α residues Gly^{193}–Lys^{215} containing an AMS-modified Cys^{208} (Fig. 1, D, upper panel, and E, upper panel). For further confirmatory evidence, we synthesized this peptide, treated it with AMS, and performed
HPLC/MALDI-TOF MS analysis (Fig. 1F). The synthetic peptide was eluted at the same elution time as the peak marked # and had an identical molecular mass. The corresponding elution peak was significantly weaker in the elution profile of digested peptides derived from the oxi band (Fig. 1, D, lower panel, and E, lower panel). An Ero1α species with reduced Cys<sup>208</sup> and Cys<sup>241</sup> was therefore generated during Ero1α-catalyzed PDI oxidation.

FIGURE 2. Specific reduction of the Cys<sup>208</sup>–Cys<sup>241</sup> disulfide of Ero1α by PDI and ERp46. A, redox states of Ero1α mutants during Ero1α-catalyzed PDI oxidation. All experiments were initiated by mixing Ero1α-Cysless or Ero1α-CyslessSS (4 μM each) with reduced PDIs (10 μM each) in air-saturated buffer at 30 °C. At 0.25 min into the reaction, the mixture was quenched with TCA, washed with acetone, and modified by AMS. The redox states of Ero1α mutants were assessed by non-reducing SDS-PAGE, followed by immunoblotting with Ero1α antibody (left panel). The right panel indicates the quantification and statistical analysis of the red fraction of Ero1α-Cysless shown in the left panel (n = 3, means ± S.D.). Note that Ero1α-CyslessSS migrates more slowly on a non-reducing gel than does Ero1α-Cysless, because of the absence of the Cys<sup>208</sup>–Cys<sup>241</sup> long range disulfide. n.s., not significant; *, p < 0.05; **, p < 0.01. B, time course of the redox state changes of Ero1α mutants during incubation of Ero1α-Cysless or Ero1α-CyslessSS (4 μM each) with reduced PDIs (10 μM each). All experiments were performed as described for Fig. 1C. The redox states of Ero1α mutants were detected as described in A, C, quantification and statistical analysis of the red fraction of Ero1α-Cysless shown in B (n = 3, means ± S.D.). D, redox states of Ero1α-Cysless during reaction with the reduced form of PDIs mutants in which a CXXC sequence in either the a or a′ domain is replaced with AXXA. All experiments were performed as described for A (left panel). The right panel indicates the quantification of red Ero1α-Cysless species shown in the left panel (n = 3, means ± S.D.). *, p < 0.01; ***, p < 0.001. E, time course of the redox state changes of Ero1α-Cysless during incubation with the reduced form of PDI active-site mutants. All experiments were performed as described in Fig. 1C, F, Quantification and statistical analysis of the red fraction of Ero1α-Cysless shown in E (n = 3, means ± S.D.). WB, Western blotting.
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Efficient Reduction of the Ero1α Cys208–Cys241 Disulfide by PDI and ERp46—We next explored whether the Cys208–Cys241 disulfide in Ero1α could be reduced selectively by PDI or non-selectively by PDI family members. To this end, we assessed how efficiently Ero1α-Cysless was converted into the red species upon reaction with separate PDI family enzymes. Whereas Ero1α-Cysless could be reduced to some extent by all tested PDIs, PDI and ERp46 were the particularly efficient producers of the red species of Ero1α within 0.25 min (Fig. 2A). Meanwhile, Ero1α-CyslessSS did not exhibit this upward band shift upon addition of any PDI family enzyme, as expected because of the lack of Cys208 and Cys241. ERp57, which has a similar overall domain arrangement to PDI, catalyzed a slower reduction of the Cys208–Cys241 disulfide than did PDI. After a 10-min incubation, however, ERp57 generated an equivalent amount of the red Ero1α species as did PDI (Fig. 2B). Statistical analysis of the time course of the change in redox state of Ero1α indicated that PDI and ERp46 produced the red Ero1α species at a faster rate than did the other tested PDIs (Fig. 2C).

We next explored whether the Ero1α Cys208–Cys241 pair has a preference for thiol-disulfide exchange with the PDI a’ domain active site or the a domain active site. For this purpose, Ero1α-Cysless was incubated with two different PDI mutants in which the redox active site cysteines in either the a or a’ domain were replaced by alanines. The resulting constructs were named PDI AXAX-CXXC and PDI CXXC-AXXX, respectively. PDI AXAX-CXXC reduced Ero1α-Cysless more efficiently than PDI CXXC-AXXX (Fig. 2D), indicating that the C-terminal a’ domain serves as a more efficient reductant of the Ero1α Cys208–Cys241 disulfide than the N-terminal a domain. The time course of the change in the Ero1α redox state confirmed the higher reactivity of PDI AXAX-CXXC with the Ero1α Cys208–Cys241 disulfide compared with PDI CXXC-AXXX (Fig. 2, E and F). These results were consistent with our previous observation of mixed disulfide complexes trapped in the ER of living cells (18).

Role of the Cys208–Cys241 Disulfide in the Overall Structure of Ero1α—In general, there are three functions of protein disulfide bonds: structural disulfides that stabilize the conformation of proteins (24–27), catalytic disulfides that directly engage in redox reactions, and regulatory disulfides that modulate enzymatic activities (3, 4). To investigate the effect of the Cys208–Cys241 disulfide on the conformational stability of Ero1α, we performed differential scanning calorimetry (DSC) measurements for Ero1α-AA, Ero1α-AASS, Ero1α-WT, and Ero1α-WTSS at scan rates of 60, 120, and 200 °C h−1 (Fig. 3A and supplemental Fig. S1, A and C). Although Ero1α appears to assume a single-domain globular fold, all constructs displayed two or more melting points during heat denaturation, indicating a non-2-state denaturation process for Ero1α. Thermodynamic parameters of this denaturation were calculated by fitting a non-2-state model using ORIGIN software (Origin Lab) (Fig. 3B, supplemental Fig. S1, B and D, and Table 1). The denaturation of Ero1α involved two melting temperatures, Tm1 and Tm2, a three-state model that included native, partially denatured and fully denatured states was the best fit. Ero1α-AASS showed a 3.8 °C higher Tm1 value than Ero1α-AA (Table 1), and a significant increase in Tm1 was observed with the double mutant C208S/C241S in the presence of Ero1α-WT-based constructs (i.e. Ero1α-WTSS versus Ero1α-WT). Additionally, Ero1α-WT-based constructs tended to have higher Tm1 values than Ero1α-AA-based constructs, indicating that the presence or absence of disulfide bonds in loop I and loop II influences the first thermal denaturation step of Ero1α. By contrast, Tm2 was affected to a much lesser extent by disulfide bonds in loops I and II (Table 1), suggesting that the second denaturation step could be ascribed primarily to the denaturation of the main a-helical domain of Ero1α. In general, the Tm2 value was comparable among all Ero1α mutants tested, regardless of the scan rate (supplemental Fig. S1, A and C, and Table 1), indicating that Ero1α retained virtually all thermodynamic stability upon deletion of the Cys208–Cys241 disulfide.

To gain further insight into the role of the Cys208–Cys241 disulfide in the overall fold of Ero1α, we carried out small angle X-ray scattering (SAXS) measurements. Fig. 4A shows the SAXS profiles of Ero1α-AA and Ero1α-AASS extrapolated to zero concentration. Guinier plots are linear without any upward curvature at low Q2 (Fig. 4A, inset), indicative of no protein aggregation. The apparent radius of gyration, Rg, and the normalized forward intensity, I(0)/c, were determined from the slope and intercept of the linear fits (supplemental Fig. S2 and Table 2). The Rg values of Ero1α-AA and Ero1α-AASS were estimated to be 26.6 ± 0.1 and 26.2 ± 0.1 Å, respectively. The molecular mass estimated from the normalized forward intensity I(0) value using BSA (66.4 kDa) as the standard reference was 54.3 kDa for both Ero1α-AA and Ero1α-AASS, suggesting that both were monomeric in solution. Furthermore, the pair distribution function, P(r), was calculated from the SAXS curves using GNOM (28), and P(r) for Ero1α-AASS was almost superimposable to that of Ero1α-AA (Fig. 4B), although the largest r value (Dmax) of Ero1α-AA and Ero1α-AASS was 96 Å and 92 Å, respectively (Table 2). These results suggest that the overall molecular shape of Ero1α was not altered by the C208S/C241S double mutation.

In our previous crystallographic study on human Ero1α, the electron density was completely missing for both loop I and loop II (Fig. 1A), indicating considerable flexibility in these two segments (21). To further analyze the conformation of Ero1α in these flexible regions, the ensemble optimization method (EOM) was performed using the SAXS data (29) and the Ero1α crystal structure (Protein Data Bank code 3AHQ) as a rigid body scaffold. Consequently, whereas a SAXS curve estimated from any certain conformation of Ero1α did not coincide with the observed curve, ensembles of multiple conformations with distinct loop I and loop II structures produced a better fit (Fig. 4C). However, it was impossible to reach an optimal ensemble of different Rg and Dmax values for both Ero1α-AA and Ero1α-AASS, because the conformational distribution did not converge into a unique pattern (Fig. 4D). Such diversity in allowed conformation ensembles suggests that Ero1α in solution is likely to be highly dynamic, particularly in the loop I and loop II regions.

Together, the thermodynamic and structural analyses indicated that the overall fold of Ero1α was not altered by mutation of the Cys208–Cys241 pair. In other words, the Cys208–Cys241 disulfide functions mainly as a regulatory disulfide that fine-
tunes the activity of Ero1α. However, the influence of its structural role that determines the thermodynamic stability of Ero1α cannot be ignored (see the next section).

Deletion of Loop II Mimics the Phenotypes of Ero1α-AASS—The fact that loop II is not conserved in yeast Ero1p suggests that Ero1 family enzymes in higher eukaryotes acquired a new mechanism of regulation through the insertion of this loop region. To explore the enzymatic role of this loop, we constructed Ero1α mutants Ero1α-AA and Ero1α-AASS, in which the Arg216–Gly239 segment corresponding to loop II was deleted in Ero1α-AA and Ero1α-AASS, respectively. We first monitored the time course of PDI oxidation by these Ero1α mutants in the absence of GSH (Fig. 5A, upper panel).

**Figure 3.** Thermal stability of Ero1α-AA and Ero1α-AASS. A, DSC curves of Ero1α-AA (2.02 mg/ml), Ero1α-AASS (1.99 mg/ml), Ero1α-WT (2.00 mg/ml), and Ero1α-WTSS (1.94 mg/ml). DSC measurements were performed at a scan rate of 60 °C/h. B, non-2-state fit of DSC curves for Ero1α-AA (upper left panel), Ero1α-AASS (upper right panel), Ero1α-WT (lower left panel), and Ero1α-WTSS (lower right panel) in A.

**Table 1.**

| Scan rate | 60 °C/h | 120 °C/h | 200 °C/h |
|-----------|--------|---------|---------|
|           | Tm1    | ΔH1 (Tm1) | Tm2    | Tm1 | ΔH1 (Tm1) | Tm2 | Tm1 | ΔH1 (Tm1) | Tm2 |
| Ero1α-AA  | 46.9 ± 0.1 | 217 ± 7  | 58.7 ± 0.1 | 132 ± 2 | 46.7 ± 0.3 | 157 ± 19 | 60.0 ± 0.1 | 141 ± 9 | 46.2 ± 0.6 | 159 ± 15 | 61.3 ± 0  | 162 ± 6 |
| Ero1α-AASS| 50.7 ± 0.3 | 248 ± 30 | 57.4 ± 0  | 133 ± 8 | 51.3 ± 0.1 | 203 ± 28 | 58.7 ± 0.1 | 149 ± 5 | 49.6 ± 0.6 | 190 ± 57 | 60.4 ± 0  | 143 ± 9 |
| Ero1α-WT  | 51.3 ± 0.2 | 230 ± 20 | 58.8 ± 0  | 129 ± 3 | 51.5 ± 0.1 | 232 ± 20 | 60.0 ± 0.1 | 134 ± 2 | 52.3 ± 0.2 | 202 ± 5  | 61.1 ± 0  | 131 ± 1 |
| Ero1α-WTSS| 53.8 ± 0  | 166 ± 1  | 58.4 ± 0.1 | 158 ± 2 | 54.2 ± 0.1 | 203 ± 4  | 59.4 ± 0  | 151 ± 1 | 54.7 ± 0.1 | 244 ± 9  | 60.4 ± 0  | 142 ± 1 |

Deletion of Loop II Mimics the Phenotypes of Ero1α-AASS—The fact that loop II is not conserved in yeast Ero1p suggests that Ero1 family enzymes in higher eukaryotes acquired a new mechanism of regulation through the insertion of this loop region. To explore the enzymatic role of this loop, we constructed Ero1α mutants Ero1α-ΔAA and Ero1α-ΔAASS, in which the Arg216–Gly239 segment corresponding to loop II was deleted in Ero1α-AA and Ero1α-AASS, respectively. We first monitored the time course of PDI oxidation by these Ero1α mutants in the absence of GSH (Fig. 5A, upper panel). Ero1α-AASS oxidized PDI with higher efficiency than did Ero1α-AA (Fig. 5B and supplemental Fig. S3). Noticeably, both Ero1α-ΔAA and Ero1α-ΔAASS oxidized PDI faster than Ero1α-AA, indicating that deletion of loop II enhanced the PDI oxidation activity of Ero1α. Thus, these mutants...
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A

\[ \ln(I(Q)(\text{a.u.})) = \frac{1}{Q^2} + b \]

Inset:

\[ Q_r^2 (\text{Å}^2) \]

Ero1α-AA
Ero1α-AASS

B

P(r)(a.u.)

0 20 40 60 80 100

r (Å)

Ero1α-AA
Ero1α-AASS

C

Ero1α-AA

Experimental SAXS curve
Fitting theoretical SAXS curve

Loop I
Residues 166-172
N-terminal region
Crystal structure
3AHQ (45.685)

(3.583)

(2.397)

EOM (1.422)

Ero1α-AASS

Experimental SAXS curve
Fitting theoretical SAXS curve

Loop I
Residues 166-172
N-terminal region
Crystal structure
3AHQ (42.875)

(9.848)

(2.648)

EOM (2.270)

D

Ero1α-AA

Loop I
Loop II
Residues 166-172
N-terminal region
Crystal structure

D_{max} (Å)

Fraction

R_g (Å)

Ero1α-AASS

Loop I
Loop II
Residues 166-172
N-terminal region
Crystal structure

D_{max} (Å)

Fraction

R_g (Å)
likely adopt a conformation similar to that of the hyperactive Ero1α-AASS (18) (see “Discussion”).

As previously reported (23), the presence of GSH dramatically changes the ranking of the oxidative activity of Ero1α mutants; under conditions where reduced PDI is constantly regenerated by GSH, NADPH consumption driven by Ero1α-AASS was not faster but rather slower than that by Ero1α-AA (Fig. 5, A, lower panel, and C). Again, both Ero1α-ΔAA and Ero1α-ΔAASS displayed similar kinetics to Ero1α-AASS; mutants lacking loop II oxidized PDI more slowly than Ero1α-AA in the presence of GSH.

We also measured O2 consumption and H2O2 production in the absence and presence of GSH. These assays also demonstrated that although the Ero1α mutants lacking loop II oxidized PDI faster and hence generated higher levels of H2O2 than Ero1α-AA in the absence of GSH (Fig. 5, D, left panel), and E, left panel), the opposite was true in the presence of GSH; Ero1α-AA consumed O2 fastest and hence produced the largest amount of H2O2 among the Ero1α derivatives tested (Fig. 5, D, right panel, and E, right panel).

To address the mechanistic implication of loop II on Ero1α activity in the presence of GSH, we analyzed the redox state of Ero1α in solution. As expected, a significant portion of Ero1α-AA was reduced by PDI in the presence of GSH (Fig. 6A, left panel), but this reduction was significantly delayed with Ero1α-ΔAA (Fig. 6A, right panel). Thus, deletion of loop II inhibited PDI-mediated reduction of the Cys208–Cys241 disulfide. Given that the redox interaction between PDI and the Cys208–Cys241 pair is rate-limiting under such reductive conditions (23), this finding likely explains the compromised catalytic activity of Ero1α-ΔAA in the presence of GSH (Fig. 5, C–E; see also “Discussion”). Furthermore, in the absence of GSH, Ero1α-ΔAA was reduced by PDI to a much lesser extent compared with Ero1α-AA (Fig. 6B, left panel), as was Ero1α-ΔCysless compared with Ero1α-Cysless (Figs. 2B and 6B, right panel). These results suggest that loop II plays a significant role in the efficient reduction of the Ero1α Cys208–Cys241 disulfide by PDI. However, in the absence of GSH, loop II deletion mutants appear to adopt an active, Ero1α-AASS-like conformation that is independent of the redox state of the Cys208–Cys241 pair (see “Discussion”).

**Discussion**

Our previous study demonstrated that the human Ero1α C104A/C131A/C208S/C241S quadruple mutant oxidizes PDI more rapidly and therefore produces more H2O2 than does Ero1α C104A/C131A with the Cys208–Cys241 disulfide intact. As a result, cell proliferation is greatly inhibited, and cell viability is significantly diminished (18), indicating a physiologically significant role in regulation for Ero1α Cys208 and Cys241. Consistent with this, the Cys208–Cys241 pair is highly conserved in Ero1 family enzymes in higher eukaryotes, although not in the yeast enzyme Ero1p (4).

The present study revealed that mutation of Cys208 and Cys241 has little effect on the thermodynamic stability and overall shape of Ero1α (Figs. 3 and 4B, supplementary Fig. S1, and Tables 1 and 2), leading us to conclude that the Cys208–Cys241 disulfide functions mainly as a regulatory element that fine-tunes the Ero1α activity. The Cys208–Cys241 disulfide was preferentially reduced by two members of the PDI family; PDI and ERp46 (Fig. 2, A–C). In this context, our previous studies indicated that Ero1α is capable of selectively oxidizing PDI and, to a lesser extent, ERp46 (5), although Ero1α is incapable of efficiently oxidizing other members of the PDI family (30). The present study also showed that the Cys208–Cys241 disulfide was reduced by the PDI a’ domain more efficiently than by the a domain (Fig. 2, D–F). In line with this, Ero1α preferentially oxidizes the PDI a’ domain and oxidizes the a domain to a lesser extent (30, 31). Thus, there seems to be a close correlation between the reactivity of PDIs with the Ero1α Cys208–Cys241 disulfide and the selective oxidation of PDIs by the Ero1α active site Cys94–Cys99.

**FIGURE 4. Overall shape of Ero1α-AA and Ero1α-AASS in solution.** A, SAXS profiles of Ero1α-AA (blue) and Ero1α-AASS (red). The inset shows Guinier plots of Ero1α-AA and Ero1α-AASS from Guinier analysis using the Q range (highlighted data points in the inset) shown in Table 2. B, pair distribution function P(r) of Ero1α-ΔAA (blue) and Ero1α-AASS (red). C, curves drawn with black dots correspond to the experimental SAXS curves of Ero1α-ΔAA (left panel) and Ero1α-AASS (right panel). Each experimental SAXS curve is offset for clarity of presentation. The values in parentheses represent χ2 values resulting from fitting of the theoretical SAXS curve (red) to the experimental data (black dots). The data are best explained using the EOM distribution of Rg and the largest r value, Dmax, for Ero1α-ΔAA (left panel) and Ero1α-AASS (right panel) predicted by EOM analysis. Representative models of members of the conformational ensemble are shown. The modeled structures are composed of the Ero1α crystal structure with a rigid body (cyan) and putative flexible loops that are missing in the crystal structure.

**TABLE 2**

| Oligomeric state | R^a | R^b | I(0)^c | I(0) | Dmax^d | 
|------------------|-----|-----|--------|------|--------|
| Monomer          |     |     |        |      |        |
| Monomer          |     |     | Monomer|      |        |

* Guinier analysis using the Q range from 0.01562 to Qmax < 1.3/Rg.
* Estimates in real space upon P(r) determination.
* Maximum dimension estimated using the GNOM package.
* Porod volume.
* Molecular mass calculated using the I(0) value for BSA as the standard.
* Molecular mass calculated according to empirical relationship MM = Mv/1.65 (42).
* Theoretical molecular mass calculated according to amino acid sequences.
* Extrapolated to infinite dilution.
* At a concentration of 2.75 mg/ml (39).
* ND, not determined.

**FIGURE 5. Overall shape of Ero1α-ΔAA and Ero1α-ΔAASS in solution.** A, SAXS profiles of Ero1α-ΔAA (blue) and Ero1α-ΔAASS (red). The inset shows Guinier plots of Ero1α-ΔAA and Ero1α-ΔAASS from Guinier analysis using the Q range (highlighted data points in the inset) shown in Table 2. B, pair distribution function P(r) of Ero1α (blue) and Ero1α-AASS (red). C, curves drawn with black dots correspond to the experimental SAXS curves of Ero1α-ΔAA (left panel) and Ero1α-AASS (right panel). Each experimental SAXS curve is offset for clarity of presentation. The values in parentheses represent χ2 values resulting from fitting of the theoretical SAXS curve (red) to the experimental data (black dots). The data are best explained using the EOM distribution of Rg and the largest r value, Dmax, for Ero1α-ΔAA (left panel) and Ero1α-AASS (right panel) predicted by EOM analysis. Representative models of members of the conformational ensemble are shown. The modeled structures are composed of the Ero1α crystal structure with a rigid body (cyan) and putative flexible loops that are missing in the crystal structure.
In this study, we demonstrated that reduction of the Cys208–Cys241 disulfide increased the oxidative activity of Ero1α/H9251 (Figs. 5 and 6, A and B). As shown in supplemental Fig. S4 (B and D), the proportion of Ero1α with a reduced Cys208–Cys241 pair increased with absolute levels of reduced PDI. We also observed that the Cys208–Cys241 disulfide could be reduced more effectively by a combination of PDI and GSH than by GSH alone (supplemental Fig. S4, A and B). Together, these results show that the differences in the reactivity of Ero1α in the presence and absence of GSH are dependent on the availability of reduced PDI or the rate of recycling of reduced PDI. In this respect, GSH may serve as an indirect enhancer of Ero1α activity.

FIGURE 5. Role of loop II in the regulation of Ero1α activity. A, schematic representation of the Ero1α-catalyzed PDI oxidation assay system in the absence (upper panel) and presence of GSH, GR, and NADPH (lower panel). B, fractions of reduced (red) PDI (left panel), partially oxidized (oxi) PDI (middle panel), and oxidized (oxi) PDI (right panel) in supplemental Fig. S3 were quantified using an LAS-3000 image reader and plotted as a function of reaction time (n = 3, means ± S.D.). C, NADPH consumption coupled to PDI oxidation catalyzed by each Ero1α mutant. All experiments were performed in air-saturated buffer containing NADPH (200 μM), GR (1 unit), GSH (1 mM), reduced PDI (10 μM), and each Ero1α mutant (4 μM) at 30 °C. D, O2 consumption coupled to PDI oxidation catalyzed by Ero1α mutants. All experiments were initiated by mixing each Ero1α mutant (4 μM) with reduced PDI (100 μM) (left panel) or by mixing each Ero1α mutant (4 μM) with reduced PDI (100 μM) in the presence of GSH (1 mM), GR (1 unit), and NADPH (200 μM) (right panel) in air-saturated buffer at 30 °C, as shown in A. E, H2O2 generation during PDI oxidation catalyzed by Ero1α mutants. All experiments were initiated by mixing each Ero1α mutant (4 μM) with reduced PDI (100 μM) (left panel) or by mixing each Ero1α mutant (4 μM) with reduced PDI (100 μM) in the presence of GSH (1 mM), GR (1 unit), and NADPH (200 μM) (right panel) in air-saturated buffer at 30 °C, as shown in A (n = 3, means ± S.D.). Note that the H2O2 concentration decreases during the later stages of the reaction because of PDI-mediated scavenging of H2O2 as reported previously (18).
truding β-hairpin loop in Ero1α and the substrate binding pocket in the PDI b’ domain and via a sustained thiol-disulfide exchange between the loop I cysteines of Ero1α and the a’ domain active site of PDI during catalysis (32). More recently, however, we found that a non-catalytic mixed disulfide complex involving Ero1α Cys208 or Cys241 (Ero1α-PDIfast) is the predominant species detectable in the ER at steady state (18). However, whereas the Ero1α-PDIfast complex appears to be kinetically stabilized in cells, this was hardly detectable in in vitro assays, suggesting that there might be additional players that stabilize the mixed disulfide complex in cells. Similar mixed disulfide complexes were also observed with ERp57 (18), which has a very similar overall domain arrangement to PDI (33). ERp46, another preferred substrate of Ero1α, is composed of three Trx-like domains linked by unusually long flexible loops, where the three solvent-exposed redox active sites are separately located (34). These structural features of PDI and ERp46 could be advantageous for their close access to the regulatory cysteines of Ero1α, leading to the facilitated activation of Ero1α.

EOM analysis suggested that loop I and loop II are highly flexible in solution (Fig. 4D), and this flexibility is likely key to the functional redox interplay between Ero1α and PDI. Accordingly, deletion of loop II compromised the efficiency of the
Cys^{208}–Cys^{241} disulfide reduction by PDI. At this stage, the PDI b’ domain that houses the substrate binding pocket may perform an auxiliary role in another mode of the Ero1α–PDI interactivity. Specifically, Ero1α loop II might be recognized by the substrate binding pocket in the PDI b’ domain, resulting in the facilitated reduction of the Cys^{208}–Cys^{241} disulfide by the PDI a’ domain.

The presence of loop II was intrinsically inhibitory to the activity of Ero1α. Accordingly, both Ero1α–ΔAA and Ero1α–ΔAASS displayed higher PDI oxidation activity than Ero1α–AA in the absence of GSH (Fig. 5, A, upper panel, and B). Importantly, the effects on Ero1α activity caused by deletion of this loop and the substitution of Cys^{208} and Cys^{241} were neither additional nor synergistic to each other. In this context, a previous molecular dynamics simulation suggested that the removal of the Cys^{208}–Cys^{241} disulfide induces a slight but significant rearrangement of the four helices embracing the FAD moiety, resulting in the enhanced access of O₂ to the FAD isoalloxazine ring (18). Presumably, deletion of loop II perturbs the local conformation in the vicinity of the engineered site and possibly the orientation of the two helices connected by loop II, resulting in similar effects on the structure and functionality of Ero1α as occur with the C208S/C241S double mutations. As demonstrated previously (18), a loop II deletion mutant that is directly comparable with Ero1α–ΔAA was not properly expressed in human cells, possibly because of compartment-specific ionic strength, redox balance, or degradation factors. Thus, loop II appears to be essential for the stability of Ero1α in the native environment of the ER.

The loop II deletion mutants presented fundamentally different phenotypes in the presence of GSH. Under reducing conditions in which Ero1α works at maximal turnover rate (23), these mutants exhibit a defect with regard to PDI oxidation and H₂O₂ production. Our results indicate that this defect may be due to a decreased propensity for the deletion mutants to become reduced at the Cys^{208}–Cys^{241} disulfide by PDI. This defect resembles the compromised activity of Ero1α–AASS under reducing conditions, which cannot be activated by reduced PDI because of the absence of the Cys^{208}–Cys^{241} pair (23).

Collectively, our results showed that loop II provides a platform for functional interplay with reduced PDI. Consequently, our in vitro experiments establish a novel regulation mechanism of human Ero1α (Fig. 6C). During Ero1α activation, reduced PDI acts on the regulatory disulfides Cys^{94}–Cys^{131} and Cys^{99}–Cys^{104} that are located in loop I to generate active Ero1α with the Cys^{94}–Cys^{99} disulfide (35). The active Ero1α then undergoes further activation through reduction of the Cys^{208}–Cys^{241} disulfide by the reduced PDI a’ domain, possibly leading to more efficient entry of O₂ into the FAD-binding pocket. We previously reported that the four regulatory cysteines in loop I communicate with the Cys^{208}–Cys^{241} pair in loop II, either intramolecularly or intermolecularly. Furthermore, cleavage of the Cys^{208}–Cys^{241} disulfide appears to involve a thermodynamic preference for the Cys^{94}–Cys^{131} disulfide over the Cys^{94}–Cys^{99} disulfide, which stabilizes the inactive form of Ero1α (23). Thus, the Ero1α hyperactive state with the Cys^{94}–Cys^{99} disulfide and reduced Cys^{208} and Cys^{241} is presumably short-lived unless reduced PDI is continually provided. We propose that the Cys^{208}–Cys^{241} pair acts as the second PDI-mediated switch that controls Ero1α activity by somehow communicating with the four regulatory cysteines in loop I. At this stage, the availability of reduced PDI, which is representative of the redox environment in the ER, essentially regulates the disulfide bond pattern in loop I, as well as the redox state of the Cys^{208}–Cys^{241} disulfide in loop II. These findings highlight a dual regulation mechanism of mammalian Ero1α that proceeds via redox interplay between PDI and both loop I and loop II of Ero1α.

**Experimental Procedures**

**Recombinant Protein Expression and Purification**—cDNAs encoding human Ero1α and human PDIs (PDI, ERp72, ERp57, ERp46, and P5) and their mutants were subcloned into the NdeI and BamHI sites of the pET15b vector (Novagen). Proteins were overexpressed in *Escherichia coli* strain BL21 (DE3) and purified as described previously (18). Plasmids for overexpression of a set of Ero1α mutants with the deletion of the Arg^{216}–Gly^{239} segment were constructed using a PrimeSTAR mutagenesis basal kit (Takara Bio). The resultant Ero1α mutants are single-polypeptide chains in which residues 215 and 240 are covalently linked. The expression and purification of the Ero1α mutants were performed as described previously (21).

**Analysis of the Redox State of PDIs and Ero1α**—Purified PDIs were reduced with 10 mM DTT for 10 min at 4 °C, and thereafter, DTT was removed by passing the sample through a PD-10 column (GE Healthcare) pre-equilibrated with 50 mM Tris/HCl (pH 7.5) buffer containing 300 mM NaCl. The catalytic oxidation of PDIs by Ero1α was initiated by mixing 10 or 100 μM of reduced PDIs with 4 μM of each Ero1α derivative in air-saturated buffer containing 50 mM Tris/HCl (pH 7.5) and 300 mM NaCl at 30 °C. At selected time points, the reaction mixture was quenched with 5% TCA, washed with acetone, and dissolved in buffer containing 100 mM Tris/HCl (pH 7.0), 1% SDS, and 1 mM maleimide-PEG-2k or AMS. All samples were separated by non-reducing SDS-PAGE followed by staining with Coomassie Brilliant Blue (CBB) or immunoblotting with antibody. The band intensity was measured for each redox state of PDIs and Ero1α using a LAS-3000 image reader (Fujifilm).

**Statistical Analysis**—The statistical significance of differences was examined by one-way analysis of variance with Tukey honestly significant difference (HSD) post hoc testing. All statistical tests were performed using KaleidaGraph statistical software (Synergy Software) at a significance level of α = 0.05.
TOF MS (Bruker Daltonics) (36, 37). Synthetic peptides were prepared by the Fmoc (N-(9-fluorenylethoxycarbonyl) solid phase method, modified with AMS, and analyzed by HPLC and MALDI-TOF MS as described previously (5, 34, 37).

**Differential Scanning Calorimetry**—DSC measurements in 20 mM phosphate buffer (pH 7.0) were performed for Ero1α-AA and Ero1α-AASS using a MicroCal VP-capillary DSC (Malvern Instruments) at a scan rate of 60, 120, and 200 °C/h (38). The results were cubic baseline adjusted, analyzed by integration of the total area under the curve, and fitted to a non-2-state model using ORIGIN DSC analysis software (Origin Lab).

**Small Angle X-ray Scattering**—SAXS measurements were performed for Ero1α-AA, Ero1α-AASS, and BSA (Sigma-Aldrich) as a reference for determination of the molecular mass (39) in 20 mM phosphate buffer (pH 7.0) containing 150 mM NaCl and 5% glycerol at the RIKEN SPring-8 Beamline BL45XU (Hyogo, Japan) (40). For each sample, 20 SAXS images were collected using a PILATUS 3X 2 M detector (DECTRIS) at an X-ray wavelength of 1.0 Å with a camera distance of 2.0 m and an exposure time of 1 s at 20.2 °C. Analysis of SAXS data were carried out as described previously (34, 38).

**NADPH Consumption Assay**—Measurement of NADPH consumption coupled with Ero1α-catalyzed PDI oxidation was carried out at 30 °C using a U-3310 spectrophotometer (Hitachi High-Technologies). Each sample solution contained 4 μM Ero1α mutant, 10 μM PDI, 1 mM GSH, 1 unit of glutathione reductase (GR), and 200 μM NADPH (5).

**Measurement of O2 and H2O2 Levels during Ero1α-catalyzed PDI Oxidation**—Measurement of O2 consumption were performed with a FireStingO2 fiber optic oxygen meter (Pyro Science) by mixing 100 μM reduced PDI with 4 μM of Ero1α mutants in air-saturated buffer or by mixing 10 μM of reduced PDI with 4 μM of Ero1α in the presence of 1 mM GSH, 1 unit of GR, and 200 μM NADPH at 30 °C. The level of Ero1α-generated H2O2 was measured using a Pierce quantitative peroxide assay kit (Thermo Scientific) and Multiskan FC microplate reader (Thermo Scientific) as described previously (18).

**Author Contributions**—S. K. performed most of the experiments, analyzed the results, and wrote the manuscript. M. O. carried out HPLC and MALDI-TOF MS analysis and contributed to the critical reading of the manuscript. K. Y. performed DSC experiments. T. H. contributed to SAXS experiments. C. A.–H. and T. R. provided the ideas for the project. S. A. performed SAXS analysis. K. I. supervised the study and wrote the manuscript. All authors discussed the results and approved the final version of the paper.

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