Crystal structures of human Ero1 α reveal the mechanisms of regulated and targeted oxidation of PDI

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In the endoplasmic reticulum (ER) of eukaryotic cells, Ero1 flavoenzymes promote oxidative protein folding through protein disulphide isomerase (PDI), generating reactive oxygen species (hydrogen peroxide) as byproducts. Therefore, Ero1 activity must be strictly regulated to avoid futile oxidation cycles in the ER. Although regulatory mechanisms restraining Ero1 α activity ensure that not all PDIs are oxidized, its specificity towards PDI could allow other resident oxidoreductases to remain reduced and competent to carry out isomerization and reduction of protein substrates. In this study, crystal structures of human Ero1 α were solved in its hyperactive and inactive forms. Our findings reveal that human Ero1 α modulates its oxidative activity by properly positioning regulatory cysteines within an intrinsically flexible loop, and by fine-tuning the electron shuttle ability of the loop through disulphide rearrangements. Specific PDI targeting is guaranteed by electrostatic and hydrophobic interactions of Ero1 α with the PDI b'-domain through its substrate-binding pocket. These results reveal the molecular basis of the regulation and specificity of protein disulphide formation in human cells.

The EMBO Journal (2010) 29, 3330–3343. doi:10.1038/emboj.2010.222; Published online 10 September 2010

Subject Categories: proteins; structural biology

Keywords: disulphide bond; ER quality control; human Ero1 α ; redox homeostasis; X-ray crystal structure analysis

Introduction

Many secretory and membrane proteins form disulphide bonds in the endoplasmic reticulum (ER), under the assistance of numerous thiol-disulphide oxidoreductases (Sevier and Kaiser, 2006; Appenzeller-Herzog and Ellgaard, 2008; Hatahet and Ruddock, 2009). Elaborate redox networks

allow formation and isomerization of disulphide bonds until the native state is attained. The major players in oxidative reactions are protein disulphide isomerases (PDI) and Ero1; both highly conserved from yeast to mammals (Frandsen and Kaiser, 1998; Pollard *et al.*, 1998; Cabibbo *et al.*, 2000; Mezghrani *et al.*, 2001). Ero1 generates disulphide bonds *de novo* in conjunction with a flavin adenine dinucleotide (FAD) cofactor and transfers them to PDI (Tu and Weissman, 2004; Tavender and Bulleid, 2010). Protein disulphide isomerase, an ER-resident member of the thioredoxin (Trx)-fold family, is composed of two redox-active Trx domains with a CGHC motif (a- and a'-domains), two redox-inactive Trx domains (b- and b'-domains), an additional α -helical domain (c-domain) and a linker loop (x-linker) between the b'- and a'-domains. Crystal structures of yeast PDI (Pdi1p) revealed that these domains are lined up in the order a-b-b'-a'-c, and they can assume different spatial arrangements, 'twisted U-shape' or 'boat', indicating conformational flexibility of the enzyme (Tian *et al.*, 2006, 2008). Alternative conformations were also reported for human and thermophilic fungus PDIs (Nguyen *et al.*, 2008; Serve *et al.*, 2010; Wang *et al.*, 2010).

Since the discovery of an essential PDI-Ero1 oxidative pathway in *Saccharomyces cerevisiae*, its operation mechanisms have been studied using genetic, biochemical and structural approaches (Sevier and Kaiser, 2008). Recent biochemical studies demonstrated that yeast Ero1 (Ero1p) preferentially oxidizes the CXXC motif in the N-terminal Trx domain (a-domain) of Pdi1p among the active sites of oxidoreductases present in the yeast ER, leading to a preferred pathway for oxidizing the ER thiol pool (Vitu *et al.*, 2010). Furthermore, the crystal structure of Ero1p, in which its N-terminal (Thr19–Thr55) and C-terminal (Asn425–Gln560) regions were removed to obtain a high-quality protein crystal, revealed the fold of its catalytic core region and the structure of the FAD-containing reaction centre (Gross *et al.*, 2004).

The generation of each disulphide bond by Ero1p can be accompanied by the production of one molecule of hydrogen peroxide (H₂O₂), a potential reactive oxygen species (ROS) source (Gross *et al.*, 2006). Although the cell can cope with peroxides formed during routine oxidative protein folding and sometimes uses them as a messenger in the cell-signalling cascades (Veal *et al.*, 2007) and possibly as a direct protein disulphide introducer (Karala *et al.*, 2009), ROS production that exceeds the capacity of the cellular antioxidant defense systems could induce harmful ER oxidative stress. Overexpression of Ero1p was shown to cause a significant increase in ROS (Haynes *et al.*, 2004), whereas partial lowering of Ero1 activity by RNAi abrogated ROS accumulation in ER-stressed animals and enhanced resistance to the lethal effects of high levels of ER stress (Harding *et al.*, 2003; Marciniak *et al.*, 2004). These observations suggested that Ero1 activity must be tightly regulated in living cells. Yeast

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Received: 4 June 2010; accepted: 17 August 2010; published online: 10 September 2010

Ero1p, indeed, was observed to exert an inbuilt feedback regulation mechanism, in which two noncatalytic cysteine pairs (Cys90–Cys349 and Cys150–Cys295) sense the ER redox environment; their oxidation decreases Ero1p activity, thus preventing potentially futile and cell-damaging cycles and ER hyperoxidation (Sevier *et al*, 2007).

Human cells contain two Ero1 isoforms: Ero1 α that is expressed in most cell types, and Ero1 β that is found only in select tissues (Pagani *et al*, 2000; Dias-Gunasekara *et al*, 2005). While these two Ero1 isoforms seem to have selective, non-redundant functions in oxidative protein folding, the disruption of both isoforms only modestly delayed IgM production, suggesting the occurrence of Ero1-independent disulphide formation pathways in mammalian cells (Zito *et al*, 2010). Both Ero1 α and Ero1 β contain two essential cysteine triads (Cys85–Cys94–Cys99 and Cys391–Cys394–Cys397 in the case of Ero1 α), which are highly conserved from yeast to human (Bertoli *et al*, 2004). The last two cysteines in the N-terminal triad are involved in a direct interaction with PDI, whereas those in the C-terminal triad

form an active-site disulphide near the FAD isoalloxazine ring. Thus, the Cys94–Cys99 pair functions as a shuttle disulphide, transferring reducing equivalents from PDI to the FAD-containing reaction centre in Ero1 α . In contrast to yeast Ero1p, human Ero1 α was observed to oxidize the C-terminal Trx domain (a'-domain) of human PDI more effectively than the a-domain (Baker *et al*, 2008; Wang *et al*, 2009).

In living cells, human Ero1 α accumulates in two main redox isoforms with different electrophoretic mobilities on non-reducing gels (Ox1 and Ox2; Benham *et al*, 2000). Recent studies showed that these redox forms reflect disulphide bond rearrangements among Cys94, Cys99, Cys104 and Cys131, and may help modulate Ero1 α activity; Ox1 and Ox2 represents active and inactive states of the enzyme, respectively (Appenzeller-Herzog *et al*, 2008; Baker *et al*, 2008). However, yeast Ero1p lacks cysteine residues corresponding to Ero1 α Cys104 and Cys131 (Figure 1B). In the overall sequence, the amino-acid identity between human Ero1 α (468 residues) and yeast Ero1p (563 residues) is

Figure 1 Amino acid sequence, secondary structure and disulphide connectivity of human Ero1 α and yeast Ero1p. **(A)** Sequence alignment of human Ero1 α and yeast Ero1p using Clustal W. Cylinders and arrows above the sequences represent determined α -helices and β -strands in human Ero1 α , respectively. Helices constituting the four-helix bundle in the catalytic core region are shown by dark blue cylinders. Grey amino acids denote loop regions with invisible electron density. Regulatory cysteines and active-site cysteines are shown in red and green, respectively. Dotted lines connecting yellow circles indicate the characterized structural disulphides in human Ero1 α . The broken line at the N-terminal region indicates the signal sequence. **(B)** Hyperactive and inactive mutants of human and yeast Ero1s. Cysteine residues are shown with yellow circles, the Cys-to-Ala mutation sites in the Ero1 mutants are represented by grey circles. Structural, regulatory and active-site disulphides, which are based on the previous studies (Sevier *et al*, 2007; Appenzeller-Herzog *et al*, 2008; Baker *et al*, 2008), are represented by black, red and green lines, respectively. In yeast Ero1p, Cys90–Cys349 also seems to function as a regulatory disulphide because the reduction of Cys90–Cys349 and Cys150–Cys295 was observed during the Ero1p catalysis (Sevier *et al*, 2007). **(C)** Oxygen consumption by wild-type Ero1 α and its hyperactive and inactive mutants constructed in this study during the oxidation of human PDI in the presence of 10 mM reduced glutathione (GSH). A control reaction monitoring oxygen consumption in the absence of Ero1 α (GSH/PDI only) is as indicated.

approximately 27% (128 residues; Figure 1A). To elucidate the different functional regulation mechanisms in these two homologous enzymes, we set out to obtain structural information on human Ero1 α .

In this study, we report the crystal structures of full-length human Ero1 α in both its hyperactive and inactive forms, and we describe how human Ero1 α limits its oxidative activity using four regulatory cysteines properly positioned within a critical loop that transfers electrons from PDI to the FAD-containing active site. Our study provides structural and further mechanistic insights into the internal disulphide rearrangement regulating Ero1 α activity, originally proposed by the preceding studies (Appenzeller-Herzog *et al*, 2008; Baker *et al*, 2008). Our studies also suggest that the hydrophobic pocket in the PDI b'-domain is an essential element for the specific oxidation of PDI by Ero1 α and electrostatic interactions have an auxiliary role in the functional Ero1 α -PDI interaction. A detailed molecular view of the regulated Ero1 α catalysis of PDI oxidation is presented.

Results

Overall structure of human Ero1 α

High-quality crystals could not be obtained from recombinant wild-type human Ero1 α . This was probably due to its heterogeneous configurations, as suggested by non-reducing SDS-PAGE (Supplementary Figure S1). Therefore, mutants were produced in which two of the four regulatory cysteines were mutated to alanines, so that only one disulphide bond can be formed. Previous mutagenesis studies demonstrated that Cys94-Cys99 is the essential disulphide to directly engage in disulphide exchange with PDI, and the Cys104&131Ala mutant that homogeneously forms the Cys94-Cys99 disulphide is hyperactive (Baker *et al*, 2008). Accordingly, this mutant exhibited an even higher rate of oxygen consumption (i.e., PDI oxidation) than wild-type Ero1 α (Figure 1C) and migrated as a single band with a slower electrophoretic mobility than the inactive mutant as did Ox1 of cellular Ero1 α (Supplementary Figure S1; Benham *et al*, 2000).

Using this hyperactive Ero1 α mutant, we obtained a well-diffracting crystal of space group *I*222 with one molecule per asymmetric unit. The X-ray diffraction data sets were collected at the maximum resolution (2.35 Å) using the SPring-8 beamline BL44XU. The structural model was refined at 2.35 Å to an R-factor of 0.237 ($R_{\text{free}} = 0.282$) with very good stereochemistry. According to PROCHECK analysis, 99.7% of the residues were in the most favoured and the allowed regions of the Ramachandran diagram, whereas no residues were in the disallowed region (Table I).

The entire human Ero1 α chain formed a single globular domain highly rich in α -helices, in which five intramolecular disulphide bonds were identified (Cys35-Cys48, Cys37-Cys46, Cys85-Cys391, Cys208-Cys241 and Cys394-Cys397; Figure 2A and B). Thus, this crystallographic study confirms the human Ero1 α disulphide-bond pattern suggested by mass spectrometric analyses (Appenzeller-Herzog *et al*, 2008). The N-terminal segment, which had been deleted and therefore was structurally uncharacterized in the crystallographic study of yeast Ero1p (Gross *et al*, 2004), formed an anti-parallel β -hairpin containing the Cys35-Cys48 and Cys37-Cys46 disulphides (Figure 2B). This region was located opposite to the reaction centre, including the isoalloxazine ring of FAD

Table I Data collection and structure determination

	Human Ero1 α Cys104&131Ala (hyperactive)	Human Ero1 α Cys99&104Ala (inactive)
<i>Data collection</i>		
Beamline	BL44XU at SPring-8	BL44XU at SPring-8
Space group	<i>I</i> 222	<i>I</i> 222
Cell dimensions (Å)	$a = 55.0$, $b = 140.7$, $c = 144.9$ $\alpha = \beta = \gamma = 90.0^\circ$	$a = 54.7$, $b = 139.1$, $c = 143.5$ $\alpha = \beta = \gamma = 90.0^\circ$
Wavelength (Å)	0.90000	0.90000
Resolution range (Å)	41.50–2.35 (2.47–2.35)	36.01–3.07 (3.24–3.07)
No. of total observations	174 207 (24 584)	37 535 (5 172)
No. of unique reflections	23 814 (3 458)	10 434 (1 419)
Completeness (%)	99.3 (99.8)	98.7 (93.1)
$I/\sigma(I)$	21.0 (4.4)	13.8 (2.9)
Multiplicity	7.3 (7.1)	3.6 (3.6)
R_{merge}^a	0.054 (0.439)	0.063 (0.428)
<i>Refinement</i>		
Resolution range (Å)	36.27–2.35	36.01–3.07
R_{work}^b	0.237	0.224
R_{free}^c	0.282	0.303
<i>RMS deviation</i>		
Bond length (Å)	0.018	0.015
Bond angle (deg)	1.7	1.6
<i>Ramachandran analysis</i>		
Most favoured (%)	89.7	85.5
Allowed (%)	10.0	13.5
Generously allowed (%)	0.3	0.9
Disallowed (%)	0.0	0.0

The number in parentheses represent statistics in the highest resolution shell.

^a $R_{\text{merge}} = \sum_j |<I(h)> - I(h)_j| / \sum_j |<I(h)>|$, where $<I(h)>$ is the mean intensity of symmetry-equivalent reflections.

^b $R_{\text{work}} = \sum (|F_o| - |F_c|) / \sum |F_o|$.

^c $R_{\text{free}} = R$ factor for a selected subset (5%) of the reflections that was not included in earlier refinement calculations.

and its proximal disulphide Cys394-Cys397 (Figure 2A), making it unlikely that the N-terminal region is directly involved in PDI oxidation catalysis. Consistently, the removal of this region did not inhibit Ero1 α oxidative activity *in vivo* (Bertoli *et al*, 2004).

Overall, the loop regions in human Ero1 α were longer than those of yeast Ero1p. In particular, three loop regions spanning from Asp90 to Cys131 (loop A, 'regulatory loop'), Cys166 to Gln172 (loop B) and Gln212 to Glu238 (loop C) lacked electron density, probably due to their extreme flexibility (Figure 2A). All of the regulatory cysteines were contained in loop A, and therefore their locations could not be specified in the hyperactive Ero1 α . It may be that the intrinsically flexible nature of loop A is essential for electron shuttling from PDI to the FAD-proximal site of Ero1 α .

Importantly, the catalytic core regions of human Ero1 α and yeast Ero1p were nearly superimposable, with an average RMSD value for C α atoms of 0.87 Å (Figure 2C). The four-helix bundle scaffold with an interior electron-accepting cofactor is a hallmark of disulphide bond-generating enzymes widely distributed from bacteria to eukaryotes (Sevier *et al*, 2005; Inaba and Ito, 2008). In contrast, there were large structural differences between human and yeast Ero1s in the other regions (Figure 2C). Noticeably, although the

Figure 2 Overall structure of human Ero1 α . (A) Ribbon diagram of the hyperactive form of human Ero1 α . Loop segments that could not be modelled due to the lack of electron density are shown by dotted lines. The regulatory loop including Cys94, Cys99, Cys104 and Cys131 is illustrated by a red dotted line. Structural and active-site disulphides characterized in this study are represented by sticks. The four-helix bundle constituting the catalytic core region of Ero1 α is shown in dark blue. The FAD molecule is represented by balls, in which carbon, nitrogen, oxygen and phosphorus atoms are in yellow, blue, red and orange, respectively. (B) Close-up view of the N-terminal region of human Ero1 α . Disulphide bridges formed in this region are represented by sticks. Numbers indicate the residue number from the N-terminus. (C) Superimposition of human Ero1 α and yeast Ero1p. Crystal structures of the hyperactive form of human Ero1 α (magenta) and yeast Ero1p (green) are superimposed such that the RMSD between their C α atoms is minimized. Dotted square represents the four-helix bundle scaffold shared by the two Ero1s. Inset highlights the structural differences between the Ero1s, and is viewed from a different angle to clearly show the regulatory disulphide between Cys150 and Cys295 in yeast Ero1p.

Glu146–Ser161 segment in human Ero1 α assumes a four-turn α -helix and lacks a disulphide bond, the corresponding region in yeast Ero1p is highly disordered and includes the Cys150–Cys295 disulphide as a regulatory switch (Figure 2C) (Sevier *et al*, 2007). This structural difference most probably underlies the different regulatory mechanisms between the two Ero1s; in human Ero1 α , the primary switch that shuts off the enzymatic activity is positioned at a different site as described later.

FAD-binding structure of human Ero1 α

The Ero1 α crystal structure provides strong evidence that a protein disulphide bond is generated *de novo* by cooperation of the Cys394–Cys397 pair and bound FAD in human cells. Previous biochemical studies on flavin-dependent sulphhydryl oxidases (such as avian egg white oxidase and augmenter of liver regeneration) have suggested that the transient formation of a charge transfer (CT) complex and a C(4a) covalent adduct between flavin and a thiolate anion of a nearby

Figure 3 FAD-binding site in human Ero1 α . (A) FAD and its neighbouring structure in human Ero1 α . The FAD moiety and side chains of Tyr191, Cys394 and Cys397 are represented by sticks. (B) FAD-binding mode in human Ero1 α (magenta) and yeast Ero1p (green). Residues involved in aromatic ring stacking and van der Waals contact with the FAD moiety are numbered (red for human Ero1 α and green for yeast Ero1p) and shown by stick representation. (C) Proposed chemical scheme of *de novo* disulphide bond generation by the cooperation of the Cys394–Cys397 pair of human Ero1 α and FAD. (D) Accommodation of the isoalloxazine ring inside the four-helix bundle scaffold. The four-helix bundle and loop segment (residues 183–194) just above the isoalloxazine ring are shown in dark blue and light green, respectively. The β -hairpin region (residues 265–279) is removed for a clear view of the region.

cysteine is an obligatory step for disulphide bond generation (Heckler *et al*, 2008). In the redox-active centre of human Ero1 α , the S $^{\gamma}$ atom of Cys397 was separated from the flavin C(4a) atom by only 3.30 Å (Figure 3A), a possible range for CT complex and covalent adduct formation after reduction of the Cys394–Cys397 disulphide. A chemical scheme of *de novo* disulphide bond generation by human Ero1 α is therefore proposed in which Cys397 sequentially forms a CT complex and a C(4a) adduct with flavin. As a final step, Cys394 (a partner cysteine of Cys397) nucleophilically attacks the C–S bond of the adduct, yielding Cys394–Cys397 disulphide and reduced FAD (FADH $_2$; Figure 3C).

The FAD-binding modes of human and yeast Ero1s are remarkably similar, and involve the stacking of the Trp200 and His255 (His231 in yeast Ero1p) head groups between the isoalloxazine and adenine rings of FAD (Figure 3B). These two aromatic residues are universally conserved in the Ero1 and Erv2 families (Fass, 2008), in which Erv2 is another

FAD-containing sulphydryl oxidase family first identified in the yeast ER (Sevier *et al*, 2001). In addition, residues that contact oxygen atoms of the pyrophosphate moiety are highly conserved among flavoenzymes (Dym and Eisenberg, 2001). Arg287 is the representative residue in human Ero1 α , and seems to serve as a counter ion to the phosphate group similar to Arg260 in yeast Ero1p (Figure 3B).

Although Ero1 α consumes molecular oxygen as an electron acceptor for the catalysis of PDI oxidation, a channel or pathway for an oxygen molecule to the flavin is not evident in the structure. The FAD isoalloxazine ring was completely embedded within the protein portion and shielded by its own dinucleotide moiety (Figure 3D). This observation may suggest that some dynamic motion must occur for molecular oxygen to enter and reach the reaction centre of Ero1 α . In this context, we note that the extended loop spanning from Leu183 to Pro194 residues just above the FAD isoalloxazine ring (Figure 3D). It will be interesting to observe whether the

redox-dependent interaction between FAD and its neighbouring polypeptide or the PDI binding to Ero1 α modulates the conformation and dynamism of the FAD-proximal region, thereby facilitating molecular oxygen entry.

Structure and function of the inactive form of human Ero1 α

As addressed above, human Ero1 α possesses four regulatory cysteines (Cys94, Cys99, Cys104 and Cys131), pairing of which determines its PDI oxidation activity. To elucidate the molecular mechanism underlying this functional regulation, we constructed and analysed the crystal structure of an inactive Ero1 α mutant that had both Cys99 and Cys104 mutated into alanines. As a result, this mutant is likely to homogeneously contain the Cys94–Cys131 disulphide, instead of the Cys94–Cys99 disulphide that serves as a shuttle disulphide in the hyperactive form. The extremely slow oxygen consumption of this variant confirmed its gross inactivity (Figure 1C). In addition, inactive Ero1 α exhibited a single band with faster electrophoretic mobility on a non-reducing SDS gel (Supplementary Figure S1), consistent with

the fact that the Cys94–Cys131 disulphide bond is a peculiar feature of the Ox2 isoform (Appenzeller-Herzog *et al*, 2008). The crystal of the inactive Ero1 α variant belongs to the space group *I*222 with one molecule per asymmetric unit, similar to the hyperactive form. The X-ray diffraction data set was collected at the maximum resolution (3.07 Å) using the SPring-8 beamline BL44XU (Table I).

The overall structure of the inactive form was mostly superimposable on to that of the hyperactive form (Supplementary Figure S2), indicating that no gross conformational changes occur on rearrangement of the regulatory disulphide bonds. However, the electron density map around the regulatory loop was significantly affected by Cys94–Cys131 disulphide formation (Figure 4A). Although the electron density of the hyperactive form was completely invisible for the region from Asp90 to Cys131, the inactive form exhibited a significant electron density that was derived from the Cys94–Cys131 disulphide and the polypeptide segment in front of Cys94. This was most probably due to the increased constraint caused by the disulphide linkage between Cys94 and Cys131.

Figure 4 Structural basis of Ero1 α regulation. (A) Local conformational changes on disulphide rearrangement within the regulatory loop region. The Cys94–Cys131 disulphide formed in inactive Ero1 α is shown by stick representation. The electron density map is shown at the 1.0 contour level. (B) Geometry of the Cys94–Cys131 inhibitory disulphide relative to the Cys394–Cys397 active-site disulphide. The FAD moiety and side chains of Cys94, Cys131, Cys394 and Cys397 are represented by sticks. The loop region (residues 188–193) is removed for a clear view of the region. (C) Affinity measurements between human PDI and Ero1 α variants by surface plasmon resonance spectroscopy. Hyperactive (left) or inactive (right) Ero1 α variant was immobilized on a biosensor chip, and PDI at 2 (green), 4 (blue) or 8 μ M (red) was injected as analyte in the presence of 1 mM GSH and 0.25 mM GSSG. Calculated kinetic parameters for binding of human PDI to hyperactive or inactive Ero1 α are compiled in the lower panel.

Figure 5 Increased mobility of the regulatory loop restores activity of inactive Ero1 α . (A) Schematic representation of glycine-repeat insertion into the site after Cys131. (B) Oxygen consumption by inactive Ero1 α variants with different lengths of glycine-repeat insertions during human PDI oxidation in the presence of 10 mM GSH. A control reaction monitoring oxygen consumption in the absence of Ero1 α (GSH/PDI only) is as indicated.

One possible reason for the reduced activity imposed by the Cys94–Cys131 disulphide might be a lowered affinity with PDI. To examine this possibility, the interaction between Ero1 α and PDI was analysed by surface plasmon resonance (SPR) under a redox condition mimicking that in ER (GSH:GSSG ratio of 4:1). A two-state model yielded the best fit and therefore was adopted to calculate the association and dissociation rate constants using the first equilibrium constants. When hyperactive Ero1 α was immobilized, wild-type PDI showed rapid association ($2.0 \times 10^3 \text{ M}^{-1}\text{s}^{-1}$) and slow dissociation ($4.1 \times 10^{-3} \text{ s}^{-1}$) kinetics, resulting in a dissociation constant (K_D) of approximately 2.1 μ M (Figure 4C). The observed interaction is likely to reflect the reversible non-covalent association rather than the transient mixed disulphide formation, because cysteine-less PDI in which both the CXXC motifs in the a- and a'-domain were mutated to AXXA showed similar association and dissociation kinetics to wild-type PDI (Supplementary Figure S3). Notably, the inactive Ero1 α variant exhibited slightly lower affinity for PDI, with a K_D value of approximately 7.7 μ M (Figure 4C). The results suggest that rearrangement of the regulatory disulphides affects the PDI-binding ability of Ero1 α . However, the approximately 3.7-fold lowered affinity alone will not be able to explain the substantial loss of function of the inactive Ero1 α variant.

An alternative explanation could be that the process of electron shuttling from Cys94–Cys131 to Cys394–Cys397 is substantially prevented in the inactive Ero1 α variant. The crystal structure of inactive Ero1 α revealed that the sulphur atoms of Cys131 and Cys394 were separated by 12.5 Å (Figure 4B). This geometry seems unsuitable for the intramolecular electron shuttle in Ero1 α , given that the loop segment including Cys131 is not very flexible. Indeed, the region just after Cys131 assumes a short α -helix not only in the inactive but also in the hyperactive forms (Figure 4A), suggesting that the motion of Cys131 is restricted to a certain extent even upon cleavage of the Cys94–Cys131 disulphide.

To increase the mobility of the Cys131-neighbouring region, we examined the functional effects of inserting a three- or six-glycine repeat into the site immediately after Cys131 (Figure 5A). Despite of such engineering, these two constructs were predominantly in an oxidized form (Supplementary Figure S1), indicating little effects of the glycine insertion on the Cys94–Cys131 disulphide formation.

In addition, the insertions marginally affected the affinity of the inactive Ero1 α variants with PDI (Supplementary Figure S4). However, the glycine-repeat loop insertion significantly restored the PDI oxidation activity in a length-dependent manner (Figure 5B). Thus, the restricted mobility of the Cys131-neighbouring region seems to be a primary reason for the reduced activity of the mutant lacking Cys99 and Cys104.

Specific oxidation of PDI by Ero1 α

Another central issue in the Ero1 α –PDI oxidative axis is how Ero1 α specifically and effectively oxidizes PDI among the nearly 20 ER-resident PDI-family member proteins (Ellgaard and Ruddock, 2005). Although human PDI and Erp57 presumably assume a similar overall fold and three-dimensional arrangement of four thioredoxin-like domains, the latter was reported to be a poor and presumably nonphysiological substrate of Ero1 α (Mezghrani *et al*, 2001; Otsu *et al*, 2006). Noticeably, NMR structural analyses (Denisov *et al*, 2009) revealed the predominant presence of negative charges on the surface of the PDI bb'-domains; moreover, the hydrophobic pocket essential for substrate binding is located on the central cleft side of the b'-domain (Supplementary Figure S5). In contrast, human Erp57 displays a different protein surface and electrostatic potential on its bb'-domains and lacks a substrate-binding hydrophobic pocket in the b'-domain (Kozlov *et al*, 2006; Supplementary Figure S5). Furthermore, Erp57 bb'-domains are the minimal element sufficient for the complex formation with calreticulin (Russell *et al*, 2004). The sequence similarity between PDI and Erp57 is lower in the b'-domain compared with those in the a-, b- and a'-domains (Supplementary Figure S6), further suggesting a special role for the PDI b'-domain in binding Ero1 α .

To further examine this possibility, two chimeric proteins were constructed in which the b'-domain was mutually replaced between PDI and Erp57 (Figure 6A), and the Ero1 α -catalysed oxidation of these proteins was measured. Although Erp57 was a very poor substrate for Ero1 α compared with PDI, the Erp57-based chimera with the PDI b'-domain was oxidized by Ero1 α at a rate closer to wild-type PDI (Figure 6B). Conversely, the PDI-based chimera that had the Erp57 b'-domain substituted for the PDI b'-domain exhibited extremely slow oxidation by Ero1 α (Figure 6B). These results strongly suggest that the PDI b'-domain

Figure 6 Critical role of the PDI b'-domain in the specific PDI oxidation by Ero1 α . **(A)** Chimeric proteins between human PDI and ERp57 constructed in this study. Domains deriving from PDI and ERp57 are shown in pink and blue, respectively. The domain boundaries of the constructs are described in Materials and methods section. **(B)** Oxygen consumption by the hyperactive Ero1 α variant during oxidation of human PDI, ERp57 and the two chimeric proteins in the presence of 10 mM GSH. **(C)** Measurements of the affinity for hyperactive Ero1 α with PDI, ERp57 and the two chimeric proteins by surface plasmon resonance. The Ero1 α mutant was immobilized on a biosensor chip. For PDI and ERp57-based chimera, 2 μ M (green), 4 μ M (blue) or 8 μ M (red) samples were injected in the presence of 1 mM GSH and 0.25 mM GSSG as analytes. For ERp57 and PDI-based chimera, 8- (red), 16- (orange) or 32- μ M (purple) samples were injected similarly. Association and dissociation rate constants were determined using a two-state reaction model, and the kinetic parameters are summarized in the bottom panel. Data represent the means from at least four individual experiments.

has a crucial role in the specific Ero1 α catalysis of PDI oxidation.

Next, the effects of domain replacement on affinity for Ero1 α were investigated by SPR (Figure 6C). ERp57 exhibited an approximately 10-fold lower binding rate constant than that of PDI, whereas the dissociation rate constant was similar between PDI and ERp57. Consequently, the dissociation constant (K_D for Ero1 α) of ERp57 was approximately 10-fold higher than that of PDI, indicating that ERp57 has much lower affinity for Ero1 α than PDI. Intriguingly, the ERp57-based chimera displayed much faster kinetics especially in the association phase than wild-type ERp57, resulting in the K_D for Ero1 α value closer to that of PDI (Figure 6C). In contrast, the PDI-based chimera showed lower association and higher dissociation rate constants than wild-type PDI (Figure 6C), resulting in the extremely high K_D for Ero1 α value. Thus, the replacement of the b'-domain between PDI and ERp57 did substantially affect the affinity for Ero1 α . There is a clear correlation between the affinities for Ero1 α and the Ero1 α -catalysed oxidation rates of PDI, ERp57 and their chimeric proteins, implying that the differences in affinity observed by the SPR can explain the differences in reactivity with Ero1 α . Taken together, we conclude that the PDI b'-domain is a key functional element that determines the affinity and the reactivity between PDI and Ero1 α .

Contribution of electrostatic and hydrophobic interactions to the functional Ero1 α -PDI interaction

The molecular surface features of human Ero1 α revealed that a positively charged patch constituted by several basic residues, including Arg83, Arg383 and Arg387, is present near the regulatory loop (Figure 7A and B). Most Ero1-family proteins conserve positively charged residues (Arg or Lys) at the corresponding positions (Cabibbo *et al*, 2000). To examine the functional roles of this electrostatic patch in the Ero1 α -PDI interaction, we mutated the three arginines near the regulatory loop to alanine (RA) or aspartate (RD) in hyperactive Ero1 α . The Ero1 α variants lacking the positive residues exhibited grossly compromised activity (Figure 7C); the functional defect was greater in the RD than in the RA variants. These variants exhibited almost the same far UV CD spectra and redox state as hyperactive Ero1 α (Supplementary Figure S7A and B) and retained the ability to oxidize DTT although less efficiently than hyperactive Ero1 α (Supplementary Figure S7C). Collectively, these results suggest that their impaired activity reflect weakened binding to PDI rather than gross structural alterations. However, the SPR measurements revealed that both RA and RD mutants could bind PDI with slightly weakened affinity (Supplementary Figure S7D). Thus, the positively charged patch of Ero1 α is important for effective catalysis but does not seem the primary and sole interaction site for PDI.

Figure 7 Electrostatic and hydrophobic interactions mediate specificity of the Ero1 α -PDI interaction. **(A)** Electrostatic surface representation of human Ero1 α . The orientation of human Ero1 α is the same as in Figure 2A. The regulatory loop and the nearby positively charged patch in Ero1 α are indicated by a dotted line and an oval, respectively. Regions of basic potential are blue ($> 20 k_B T/e$) and acidic regions are red ($< -20 k_B T/e$). **(B)** Positively charged residues near the regulatory loop are highlighted. The side chains of Arg83, Arg383 and Arg387 are represented by sticks. **(C)** Oxygen consumption by the RD, RA and hyperactive mutants of Ero1 α during oxidation of human PDI in the presence of 10 mM GSH. NaCl concentration was fixed at 0.3 M. **(D)** Oxygen consumption by the hyperactive Ero1 α variant during oxidation of human PDI in the presence of 10 mM GSH and various concentrations of NaCl. **(E-H)** As in **(D)**, various concentrations of **(E)** Triton X-100, **(F)** ANS, **(G)** somatostatin or **(H)** mastoparan were added to the reaction buffer, whereas NaCl concentration was fixed at 0.3 M.

To gain further insights into the molecular basis of interaction between Ero1 α and PDI, we investigated the role of the hydrophobic pocket in the PDI b'-domain. High salt (NaCl) concentrations did not inhibit the Ero1 α -catalysed

PDI oxidation. Rather, NaCl enhanced the enzymatic activity of Ero1 α (Figure 7D), suggesting that hydrophobic interactions have a dominant role in mediating Ero1 α -PDI interactions in comparison to electrostatic ones. Thus, we examined

effects of amphipathic chemicals or substrate proteins known to interact with the b'-domain of PDI. Triton X-100 was previously reported to substantially decrease the interactions between the PDI b'-domain and model substrate peptides (Klappa *et al*, 1998). This detergent inhibited the Ero1 α -catalysed oxidation of PDI (Figure 7E), without significantly affecting DTT oxidation (Supplementary Figure S8). This inhibitory effect was observed also at concentrations lower than the crucial micellar concentration value (approximately 0.01%). Similar effects were obtained with 1-anilino-8-naphthalene-sulphonate (ANS, Figure 7F), a fluorescence probe that binds to hydrophobic patches on proteins (Streeter, 1965), *de facto* competing with Ero1 for hydrophobic pocket of PDI. Remarkably, somatostatin and mastoparan, which bind the hydrophobic pocket of the PDI b'-domain with K_D values of approximately 35 μ M and 130 μ M, respectively (Klappa *et al*, 1998), also inhibited PDI oxidation in a dose-dependent manner (Figure 7G and H). The greater effects of somatostatin may reflect its higher affinity for PDI. These results suggest that Ero1 α competes with the model substrates for PDI binding.

Discussion

Regulatory mechanism of Ero1 α oxidative activity

In all eukaryotes, the ER redox must be tightly controlled. This study elucidates the atomic resolution structures of full-length human Ero1 α in its active and inactive forms, revealing new insights into the mechanisms of disulphide-bond formation in human cells. Although human Ero1 α and yeast Ero1p share a four-helix bundle scaffold in the catalytic core region, and an intramolecular disulphide-shuttling mechanism, there are remarkable differences in their regulation. Thus, human Ero1 α does not contain a regulatory disulphide similar to the one (Cys150–Cys295) that in yeast Ero1p links the nonhelical cap region, containing Cys100–Cys105 (shuttle cysteines), to the helical core region, containing the active site Cys352–Cys355 (see Figure 1; Supplementary Figure S9). It is envisaged that reduction in the Cys150–Cys295 disulphide makes the shuttle cysteines even more mobile, thereby increasing Ero1p activity (Sevier *et al*, 2007). Another difference is observed in the disulphide that links the first cysteines in each active triad (Cys90–349 in yeast Ero1p and Cys85–Cys391 in human Ero1 α). Loss of the Cys90–Cys349 disulphide increased yeast Ero1p activity, although to a lesser effect than deletion of the Cys150–Cys295 pair (Sevier *et al*, 2007). In contrast, the Cys85–Cys391 disulphide in human Ero1 α resisted GSH-mediated reduction *in vivo* (Appenzeller-Herzog *et al* 2008) and its absence resulted in severe destabilization of Ero1 α structure and substantial decrease in Ero1 α activity (K Inaba, unpublished data; Benham *et al*, 2000; Bertoli *et al*, 2004; Tavender and Bulleid, 2010).

These features imply that both human and yeast Ero1s modulate their oxidative activity differently. In place of Ero1p Cys150–Cys295, human Ero1 α uses Cys104 and Cys131 to regulate the mobility of the electron-shuttle loop, and hence its overall activity. Our results confirm that the formation of Cys94–Cys131 and possibly Cys99–Cys104 disulphides yields an inactive form (Appenzeller-Herzog *et al*, 2008; Baker *et al*, 2008). Previous studies have suggested that Cys94 is the primary residue to form mixed disulphides with PDI

(Bertoli *et al*, 2004). An important issue concerns the identity of the partner cysteine of Cys94 during or after the disulphide exchange with PDI. In the hyperactive form containing the Cys94–Cys99 disulphide, Cys99 is unpaired during the redox interaction with PDI and is probably located around the middle of the regulatory loop, which seems to satisfactorily accomplish the electron shuttle from Cys94–Cys99 to the active-site disulphide Cys394–Cys397. In this regard, our previous study showed that a Cys99Ala mutant displayed weak dominant-negative activity in the *in vivo* oxidative folding of JcM chains, possibly reflecting its impaired detachment from PDI (Bertoli *et al*, 2004). It is inferred that Cys99 contributes to promoting the resolution of the PDI–Ero1 α (Cys94) mixed disulphide presumably by transferring oxidising equivalents from FAD to PDI through the redox communications between the Cys394–Cys397 and Cys94–Cys99 pairs. In contrast, Cys131, bound to Cys94 in the inactive isoform, is located at the edge of the regulatory loop and juxtaposed to a short α -helix (Ala133–Leu136). The resultant restricted mobility of Cys131 most probably underlies its low efficiency of intramolecular electron transfer, due to its inability to reach the inner active site. This idea is strongly reinforced by the observation that the increased mobility of the segment by glycine-repeat insertion after Cys131 significantly restored the PDI oxidation activity of the mutant (Figure 5B). Taken together, we propose that the oxidative activity of human Ero1 α is regulated by disulphide bond rearrangements within the regulatory loop, which modulate the ability to rapidly transfer reducing equivalents from PDI to the FAD-containing active site.

It is interesting to question how the hyperactive (Ox1) and inactive (Ox2) forms are interconverted in physiological conditions. Previous pulse-chase experiments demonstrated that the Ox2 form is intrinsically stable and predominant in living cells (Benham *et al*, 2000). More recently, the overexpression of wild-type PDI or cysteine-less (i.e. redox-inactive) PDI was observed to markedly increase the Ox1 form (Otsu *et al*, 2006). Conversely, PDI knockdown clearly diminished the Ox1/Ox2 ratio of endogenous Ero1 α (Appenzeller-Herzog *et al*, 2008). These findings suggest that PDI binding induces some conformational changes in the Ero1 α regulatory loop. Disulphide rearrangement within the loop is thereby promoted, leading to the preferred occurrence of the Ox1 form. It is conceivable that PDI acts not only as a substrate but also as a modulator that lowers the energy barrier for the Ox2–Ox1 interconversion and stabilizes Ox1 relative to Ox2. The physiological reductant essential for this disulphide rearrangement remains an important open question.

Molecular basis of the specific Ero1 α –PDI oxidative pathway

Another important insight gained in this study is the molecular mechanism of effective and specific PDI oxidation by Ero1 α in the ER of human cells. ERp57 has a high similarity to PDI in both amino-acid sequence (Supplementary Figure S6) and three-dimensional structure (Dong *et al*, 2009; Supplementary Figure S5). Nevertheless, Ero1 α is unable to effectively oxidize ERp57 (Figure 6B). ERp57 forms complexes with calnexin (CNX) or calreticulin (CRT) in the ER lumen (Oliver *et al*, 1999). It functions as a protein disulphide isomerase or reductase for glycoproteins entering the CNX

cycle, a pivotal system that assists secretory protein folding (Oliver *et al*, 1997; Ellgaard *et al*, 1999). Although CNX or CRT may prevent Ero1 α from oxidising ERp57 in physiological conditions, we observed that ERp57 intrinsically has low reactivity for Ero1 α . Accordingly, addition of CRT did not affect the kinetics of the Ero1 α -catalysed oxidation of ER57 (data not shown).

Our protein engineering approach demonstrated that substitution of the ERp57 b'-domain with that of PDI markedly enhanced the binding kinetics and reactivity of ERp57 against Ero1 α . Thus, functional association of Ero1 α with the PDI b'-domain is postulated to be necessary for effective PDI oxidation by Ero1 α . Consistently, the isolated a- or a'-domain of PDI was not effectively oxidized by Ero1 α (Supplementary Figure S10). In addition, the PDI-based chimera with the ERp57 b'-domain markedly lost its binding activity to Ero1 α (Figure 6B). On the basis of the above findings, we hypothesized that the specific interaction between the PDI b'-domain and Ero1 α has a role in activating the oxidase, possibly by inducing some conformational changes. It is also conceivable that the b'-domain has an important role in placing the a'-domain at a suitable position for oxidation by Ero1 α . In agreement with our model, recent biochemical studies have indicated that the PDI fragment composed of the b'-a' domains is the minimal element for a competent Ero1 α -PDI oxidative folding pathway (Wang *et al*, 2009).

As suggested by the different affinity for Ero1 α between PDI and ERp57, their Ero1 α -binding modes would be largely different. Indeed, altering the charge distributions on Ero1 α surface inhibited its functional interactions with PDI (Figure 7C). Moreover, the effects of salt, amphipathic chemicals and PDI substrates indicate that Ero1 α binds PDI through the hydrophobic pocket in the b'-domain. A possible interpretation of these findings might be that the electrostatic interaction has an auxiliary role in guiding the active site in the PDI a'-domain to the Ero1 α shuttle disulphide in the binary complex that is mainly owed to the close contacts between a portion of Ero1 α and the PDI hydrophobic pocket. The inhibitory effect of a model peptide was not observed for the yeast Ero1p-PDI oxidative system (Vitu *et al*, 2010), which is in line with the idea that yeast Ero1p binds PDI differently from human Ero1 α . As human Ero1 α and substrates seem to compete for PDI-binding site in the b'-domain, oxidative protein folding in human cells probably occurs in a step-wise manner: (1) reduced PDI binds Ero1 α to be oxidized; (2) oxidized PDI dissociates from Ero1 α to bind substrate proteins and (3) finally PDI introduces disulphide bonds into substrate proteins. ERp57 does not have a similar hydrophobic pocket in the b'-domain, despite it can bind a variety of proteins lacking defined secondary structure (Jessop *et al*, 2007). In this context, it is to be noted that overexpression or downregulation of ERp57 did not influence the Ox1/Ox2 ratio in endogenous Ero1 α (Appenzeller-Herzog *et al*, 2008). In short, the interaction between Ero1 α and PDI is extremely specific and well-tuned in living cells.

In summary, the findings of this study elucidate the molecular basis of regulated and specific PDI oxidation by Ero1 α . The strictly defined and regulated Ero1 α -PDI oxidative pathway should help prevent indiscriminate and futile electron flows in the ER environment crowded with numerous redox enzymes. Besides the potentially toxic effects of H₂O₂ generated as byproducts, an uncontrolled Ero1 activity

could ultimately prevent disulphide isomerization, an essential step for proteins that fold through intermediates containing non-native disulphide bonds (Jansens *et al*, 2002). Structural analysis of the Ero1 α -PDI complex is currently ongoing to further clarify how the two molecules interact and exchange electrons. A more precise description of the thiol-based redox networks in the ER and a comprehensive elucidation of their regulatory mechanisms would further increase our understanding of the molecular mechanisms of ER quality control, an exciting topic of study in present-day molecular cell biology.

Materials and methods

Preparation and crystallization of the hyperactive and inactive forms of human Ero1 α

The cDNA encoding human Ero1 α without the signal sequence was subcloned into the *NdeI*-*BamHI* site of the pET15b vector (Novagen). Ero1 α derivatives with site-directed Cys-to-Ala mutations in the regulatory loop were constructed using the Quick Mutagenesis Kit (Stratagene) with appropriate primer sets. In addition, the Cys166Ala mutation was introduced into all Ero1 α constructs to avoid aberrant formation of the disulphide-linked Ero1 α dimer. Ero1 α was overexpressed in *Escherichia coli* strain BL21 (DE3). Cells were grown at 30°C in Luria-Bertani (LB) medium containing 50 µg/ml ampicillin and 10 µM FAD and isopropyl- β -D-thiogalactoside (IPTG) was added at a final concentration of 0.5 mM at A₆₀₀ = ~0.5. After an additional 4 h of shaking, cells were collected and subsequently disrupted by micro-fluidizer (Niro Soavi PA2K) in buffer containing 50 mM Tris-HCl (pH 8.1), 0.3 M NaCl and 1 mM PMSF. After clarification of the cell lysate by centrifugation (12 000 r.p.m. for 20 min), the supernatant was loaded onto a Ni-NTA Sepharose open column (Qiagen). After washing the column with buffer containing 50 mM Tris-HCl (pH 8.1), 0.3 M NaCl and 20 mM imidazole, Ero1 α was eluted with the same buffer containing 200 mM imidazole. The eluted Ero1 α sample exhibited several bands overlapping around a monomer position on a nonreducing SDS gel, suggesting its heterogeneous configurations composed of several partially reduced/oxidized species. Eluted samples were then concentrated to 200 µl by ultrafiltration using Amicon Ultra (MWCO, 10 000; Millipore).

To prepare the fully oxidized form of Ero1 α , potassium ferricyanide was added to the Ero1 α solution at a final concentration of 20 mM, and the solution was left on ice for 20 min. This treatment generated a mix of Ero1 α species including monomer and various kinds of disulphide-linked oligomers. The sample was then loaded onto the Superdex-200 10/300 GL column (GE Healthcare) pre-equilibrated with the same buffer containing no imidazole, and monomeric Ero1 α was thereby separated from the disulphide-linked oligomers. The fractions containing monomeric Ero1 α as a main component were concentrated to 500 µl and applied onto the Mono-Q 10/100 GL column (GE Healthcare) pre-equilibrated with 50 mM Tris-HCl (pH 8.1). The sample was eluted with a linear NaCl gradient ranging from 0 to 500 mM. This final procedure was essential to fully purify monomeric Ero1 α , which exhibited a single band at a monomer position on a nonreducing SDS gel (Supplementary Figure S1). Recombinant Ero1 α thus obtained was used for functional assays, including oxygen consumption and SPR measurements. Purified Ero1 α exhibits the maximum absorbance around 450 nm due to the bound FAD, and an extinction coefficient of 12.5 mM⁻¹ cm⁻¹ at 454 nm was used to quantify the concentration of FAD-bound Ero1 α (Gross *et al*, 2006). Determination of total protein concentration by the bicinchoninate (BCA) method revealed that our preparation of FAD-bound Ero1 α was more than 90 % pure.

To crystallize human Ero1 α , a protein surface-engineering technique was used as follows. Lysines on the surface of our recombinant Ero1 α preparation were methylated as described previously (Walter *et al*, 2006). The methylated sample was further purified by a Superdex 200 column pre-equilibrated with 50 mM Tris-HCl (pH 8.1) and 150 mM NaCl, concentrated to approximately 10 mg/ml, and then dialysed against water. Methylated Ero1 α crystals appeared within a week by vapour diffusion at 20°C. The reservoir conditions were: 8 % PEG 4000 and 50 mM imidazole

(pH 8.0) for the hyperactive form, and 20% PEG1500 and 80 mM imidazole (pH 8.0) for the inactive form. For cryoprotection, the crystals were transferred directly to a 16% glycerol, 100-mM imidazole (pH 8.0) solution containing 20% PEG 4000 (for the hyperactive form), or 30% PEG 1500 (for the inactive form), and flash-frozen in cold nitrogen gas from a cryostat (Rigaku, Japan).

Crystallographic analysis

Diffraction data were collected at the Osaka University beamline BL44XU at SPring-8 equipped with MX225-HE (Rayonix), which is financially supported by the Academia Sinica and National Synchrotron Radiation Research Center (Taiwan, ROC). Data were integrated with HKL2000 (Otwinowski and Minor, 1997), and the crystallographic parameters have been summarized in Table 1. Crystals contained one molecule in an asymmetric unit for both hyperactive and inactive Ero1 α . Phase determination was made by molecular replacement using the published structure of yeast Ero1p (PDB code 1RP4) as the search model, using the program MOLREP (Vagin and Teplyakov, 1997). The initial structural model of human Ero1 α was constructed using the program ARP/warp (Perrakis *et al*, 2001), and was refined by several cycles of manual rebuilding and refinement using Coot (Emsley and Cowtan, 2004) and Refmac 5 (Collaborative Computational Project, 1994).

Construction of chimeric proteins between PDI and Erp57

To construct chimeric proteins in which the b'-domain was mutually swapped between PDI and Erp57, *KpnI* and *SacI* restriction sites were introduced at the start (Leu234 for PDI and Phe241 for Erp57) and end (Leu355 for PDI and Leu365 for Erp57) of the b'-domains (Supplementary Figure S4). The *KpnI*-*SacI* fragment of PDI was then inserted into the corresponding sites of the vectors encoding the a-, b- and a'-domains of Erp57. The PDI-based chimera with the Erp57 b'-domain was prepared in a similar manner. The introduced restriction sites were mutated back to their original sequences using a Quick Mutagenesis Kit with appropriate primer sets. Chimeric proteins, including wild-type PDI and Erp57, were overexpressed in *E. coli* strain BL21(DE3). The LB medium containing 50 $\mu\text{g ml}^{-1}$ ampicillin was incubated at 37°C until IPTG was added to the medium at a final concentration of 0.5 mM at $A_{600} = \sim 0.5$. After an additional 4 h of shaking at 30°C, cells were collected. The cell lysate supernatant was applied to the Ni-NTA Sepharose open column. Fractions eluted with 200 mM imidazole were further purified by anion exchange chromatography with a MonoQ column. Each construct was quantified using the BCA method.

Oxygen consumption assay

Oxygen consumption was measured using a Clark-type oxygen electrode (YSI 5331). All experiments were performed at 30°C in air-saturated buffer (approximately 235 $\mu\text{M O}_2$) in 50 mM Tris-HCl (pH 8.1) and 150 mM NaCl. Catalytic oxygen consumption was initiated by adding each Ero1 α variant to a final concentration of 2 μM in a reaction mixture containing 10 μM PDI or its derivatives and 10 mM GSH.

SPR measurements

The association and dissociation rate constants (k_{on} or k_{off}) for the direct binding of PDI, Erp57 or their chimeric proteins to

immobilized Ero1 α were determined by SPR measurements on a BIACORE2000 system (GE Healthcare). The hyperactive or inactive form of Ero1 α was coupled to the CM5 sensor chip (GE Healthcare) using amine-coupling chemistry. As a control, one channel was coupled with bovine serum albumin (BSA) to exclude background binding. Before monitoring analyte binding, immobilized Ero1 α was activated with 2 μM reduced PDI dissolved in a buffer containing 10 mM GSH. Sensorgrams were recorded for three concentrations of each analyte sample (2, 4 and 8 μM for PDI and Erp57-based chimera; and 8, 16 and 32 μM for Erp57 and PDI-based chimera) at 25°C for a 2-min association phase, followed by a 4-min dissociation phase. The running buffer was 20 mM HEPES-NaOH (pH 7.4), 150 mM NaCl, 0.001% Tween-20, 2 mM EDTA, 1 mM GSH and 0.25 mM GSSG. All analyte samples were exchanged and diluted into this buffer previously. We verified by oxygen consumption assay that the presence of 0.001% Tween-20 only modestly inhibited Ero1 α -catalysed PDI oxidation (Supplementary Figure S11). Sensorgrams were analysed by nonlinear regression analysis according to a two-state model using the BIAevaluation 4.1 software. Experiments were replicated at least four times.

Accession numbers

The coordinates and structural factors described in this study have been deposited in the Protein Data Bank with ID code 3AHQ for the hyperactive form of human Ero1 α and 3AHR for the inactive form of human Ero1 α .

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

Acknowledgements

We thank Kazutaka Araki and Kazuhiro Nagata for stimulating discussion and helpful advice on the oxygen consumption and SPR measurements; Eiki Yamashita and Atsushi Nakagawa for help with diffraction data collection; and Claudio Fagioli and Akiko Sato for technical support. We also thank Tomohisa Horibe and Masakazu Kikuchi for kindly providing the Erp57 expression vector. This study was supported by a Grant-in-Aid for Young Scientists (A) from MEXT and the Yamada Science Foundation (to KI), AIRC, Fondazione Cariplo and Telethon (to RS) and by the Targeted Proteins Research Program (TPRP) from MEXT (to MS).

Author contributions: KI carried out most of the crystallographic works and a portion of the functional analyses; SM and HI performed most of the functional analyses; SV and RS greatly contributed to editing the paper. MS carried out diffraction data analysis and structure refinement. All authors discussed the results and commented on the paper. KI supervised the work and wrote the paper.

Conflict of interest

The authors declare that they have no conflict of interest.

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