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FORUM REVIEW ARTICLE

Structure, Mechanism, and Evolution of Ero1 Family Enzymes

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Abstract

Significance: Disulfide bond formation is an essential reaction involved in the folding and maturation of many secreted and membrane proteins. Both prokaryotic and eukaryotic cells utilize various disulfide oxidoreductases and redox-active cofactors to accelerate this oxidative reaction, and higher eukaryotes have diversified and refined these disulfide-introducing cascades over the course of evolution. ***Recent Advances:*** In the past decade, atomic resolution structures have been solved for an increasing number of disulfide oxidoreductases, thereby revealing the structural and mechanistic basis of cellular disulfide bond formation systems. ***Critical Issues:*** In this review, we focus on the evolution, structure, and regulatory mechanisms of endoplasmic reticulum oxidoreductin 1 (Ero1) family enzymes, the primary disulfide bond-generating catalysts in the endoplasmic reticulum (ER). Detailed comparison of Ero1 with other oxidoreductases, such as Prx4, QSOX, Erv1/2, and disulfide bond protein B (DsbB), provides important insight into how this ER-resident flavoenzyme acts in a regulated and specific manner to maintain redox and protein homeostasis in eukaryotic cells. ***Future Directions:*** Currently, it is presumed that multiple pathways in addition to that mediated by Ero1 cooperate to achieve oxidative folding of many secretory and membrane proteins in mammalian cells. The important open question is how each oxidative pathway works distinctly or redundantly in response to various cellular conditions. *Antioxid. Redox Signal*. 16, 790–799.

Introduction

Almost all organisms, from bacteria to humans, are equipped with enzymes and redox compounds involved in generating disulfide bonds and introducing them into newly synthesized polypeptides to foster productive folding (5, 11, 14, 29). In most cases, disulfide formation is an enzymatically catalyzed process that relies on specific oxidative cascades present in the periplasm of prokaryotes, the endoplasmic reticulum (ER), or mitochondrial intermembrane space of eukaryotes. In this review, we focus on the structure, mechanism, and evolution of a group of ER-resident oxidases, the ER oxidoreductin 1 (Ero1) family (50), while its physiological aspects are addressed in more details by Ramming and Appenzeller-Herzog (46) in the same Forum. Accumulating knowledge about this flavoenzyme has greatly advanced our understanding of how protein disulfide bond formation proceeds in a regulated and specific manner in eukaryotic cells. Detailed analyses of Ero1 and other disulfide-generating catalysts in different cellular compartments and species provide an evolutionary perspective of the cellular oxidative protein folding systems.

Overview of Ero1 and Related Catalysts in the Disulfide Bond Formation Pathways of Eukaryotes

In eukaryotes, the ER provides a relatively oxidizing environment with a much lower reduced glutathione (GSH)/oxidized glutathione (GSSG) ratio than that of the cytosol (2). This environment is suitable for the oxidation of free sulfhydryl (SH) groups to form disulfide (S–S) bonds. The discovery of an ER-resident flavoprotein, Ero1p in yeast, was a turning point in ER redox biology (22, 45). Ero1, which has two paralogs (Ero1α and Ero1β) in higher eukaryotes, manufactures a disulfide bond in concert with flavin adenine dinucleotide (FAD) and serves as the primary oxidase of protein disulfide isomerase (PDI). Importantly, when oxygen is used as an electron acceptor of dithiols, hydrogen peroxide (H_2O_2), a source of reactive oxygen species (ROS), is produced as a byproduct (24). Since Ero1-mediated PDI oxidation accompanies H_2O_2 generation, Ero1 activity is tightly regulated to avoid ER hyperoxidation. This regulation is accomplished by the isomerization/reduction of noncatalytic disulfide bonds, a process in which PDI most likely plays a central role (48, 50).

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Whereas overexpression of Ero1p in yeast caused a significant increase of ROS in strains that overexpress misfolded proteins or are deficient in unfolded protein response activation (2, 27), overexpression of human Ero1α (even a deregulated variant) in HEK293 cells only slightly triggered ER stress (3), suggesting the existence of a competent antioxidant defense system in higher eukaryotes. Ero1α is expressed ubiquitously in multiple human tissues (12), whereas Ero1β is expressed more exclusively in secretory tissues, such as the pancreas (15). Ero1β exhibits a selective and nonredundant function in the oxidative folding of pro-insulin in the islets of Langerhans (60). Several lines of evidence suggest that Ero1α is transcriptionally upregulated by hypoxia-inducible factor 1 (HIF-1) or CHOP, while transcription of Ero1β is regulated directly by PDX1, a key pancreatic transcription factor (38, 44), indicating their distinct biological roles.

Unexpectedly, homozygous mice with nearly complete loss-of-function mutations in both Ero1 isoforms ($Ero1\alpha^{i/i};\beta^{i/i}$) are viable (60), while Ero1 is essential in *Saccharomyces cerevisiae* and *Caenorhabditis elegans*. This observation implies the existence of Ero1-independent oxidative pathways in mammalian cells. In this context, several alternative oxidative cascades have been reported recently. For example, an ER-localized peroxiredoxin (Prx) isoform, Prx4, metabolizes H_2O_2 by reducing it to a water molecule (51). Oxidized Prx4, in turn, could engage in the oxidation of PDI family members, ensuring continuation of the Prx4-mediated oxidative cycle (52, 61). In addition, Erv/augmenter of liver regeneration (ALR) family members, quiescin sulfhydryl oxidases (QSOX), vitamin K 2,3-epoxide oxidoreductase (VKOR), and ER-resident glutathione peroxidase (GPx) family members (GPx7/8) could function as alternative catalysts to directly catalyze disulfide bond formation in mammalian cells (11, 53, 61) (see also Table 1). Even direct oxidation by low-molecular-weight compounds such as H_2O_2, dehydroascorbate, tocopherol, and vitamin K may function as alternative pathways (41). Thus, higher organisms likely rely on a much more diversified web of oxidants than do yeast and worms.

Evolution of Eukaryotic Disulfide-Generating Catalysts

To gain an evolutionary perspective, we searched and statistically analyzed homologous genes of the above named ER-localized/related disulfide-generating catalysts using the KEGG database (www.genome.jp/kegg/catalog/org_list.html; see Supplementary Table S1; Supplementary Data are available online at www.liebertonline.com/ars) (35). As shown in Figure 1, these enzymes differentiated during evolution, especially after the emergence of the animal (metazoan) kingdom. Assuming that they have acquired functions that are redundant or complementary to each other, the nonlethality of $Ero1\alpha^{i/i};\beta^{i/i}$ mice may not be surprising.

As expected, the Ero1 family is most highly conserved ER-localized disulfide-generating catalyst in eukaryotes. At least one Ero1 family member is present in almost all eukaryotes (133 out of 143 species; see Supplementary Table S1). Two paralogs in mammals, Ero1α and Ero1β, appear to have divided into two classes after being duplicated in vertebrates, as demonstrated by the neighbor-joining method (39) (Fig. 2A). Intriguingly, some vertebrates, including panda, horse,

Table 1. Selected Disulfide-Generating Catalysts in the Oxidative Protein Folding Pathways

	Cofactor	*Domain organization*	*Localization*	*Structural data*
Ero1	FAD	ERO1	ER	Yeast wt (1RQ1, 1RP4) active (3M31) form
			(plasma membrane)	Human active (3AHQ), inactive (3AHR) form
QSOX	FAD	Trx(s) HRR Erv/ALR domain	(ER), Golgi Secretory compartment (plasma membrane)	Human QSOX1 (3LLK, 3LLI)
Erv/ALR	FAD	Erv/ALR domain		Human ALR (3O55, 3MBG)
		Mitochondria	Arabidopsis Erv1 (2HJ3)	
		ER	Rat Erv1 (1OQC), Yeast Erv2p (1JR8, 1JRA)	
Prx4	—	Trx-like domain with a TXXC motif	ER Secretory compartment Secreted (plasma membrane)	Human homodecamer (2PN8)
GPx7/8	—	Glutathione peroxidase	ER GPx8: ER membrane	Human GPx7 (2P31), GPx8 (3CYN)
VKOR	Quinone	VKOR (DsbA-like Trx) (Epimerase)	ER membrane Bacterial periplasm membrane	Homolog from *Synechococcus* sp. (3KP9, 3KP8)
DsbB	Quinone	DsbB	Bacterial periplasm membrane	DsbA-DsbB complex (2HI7, 2ZUP, 3E9J) DsbB-Fab complex (2ZUQ) NMR structure of DsbB (2K73, 2K74)

Most of the domain annotations are taken from the Pfam database. Parentheses in the Localization column suggest some exceptions. Each four-digit code in the Structural data column shows the PDB ID.

ALR, augmenter of liver regeneration; ER, endoplasmic reticulum; Ero1, endoplasmic reticulum oxidoreductin 1; FAD, flavin adenine dinucleotide; HRR, helix-rich region; Prx, peroxiredoxin; PDB, protein database; QSOX, quiescin sulfhydryl oxidases; Trx, thioredoxin; VKOR, vitamin K 2,3-epoxide oxidoreductase.

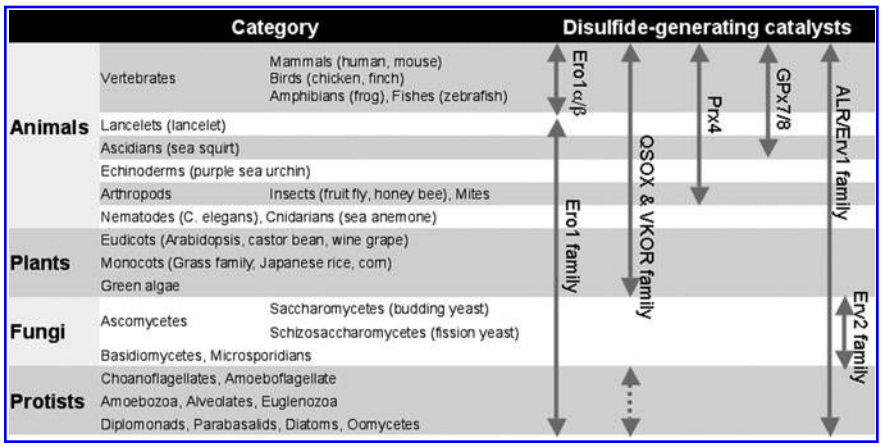

FIG. 1. Evolutional perspective of selected disulfide-generating catalysts from selected eukaryotes. *Arrows* indicate the species that code each gene. The *dashed arrow* denotes that QSOX and VKOR family genes are not completely conserved among protists. See also Supplementary Table S1 for the complete dataset. QSOX, quiescin sulfhydryl oxidases; VKOR, vitamin K 2,3-epoxide oxidoreductase.

opossum, and clawed frog, lack either Ero1α or Ero1β (Fig. 2B), suggesting that they are physiologically complementary. Indeed, all of the catalytic and regulatory cysteine residues are present in both Ero1α and Ero1β, and there are no apparent distinctive domain insertions/deletions in either (Fig. 3). Even in Ero1 genes from vertebrates lacking one of them, no characteristic features are found in their primary sequences (data not shown). While striking differences exist in transcription regulation or tissue distribution (see the above section), Ero1β may be activated under similar ER redox conditions by similar mechanisms as Ero1α. By contrast, some species, including *Arabidopsis thaliana* endoplasmic reticulum oxidoreduclin1 (AEROs) and *Schizosaccharomyces pombe* (SpEros), have more than two distinct isoforms (17, 37). Similar to human Ero1s, plant AEROs possesses different transcriptional regulatory elements (17), although the biological significance of these isoforms is poorly understood. More notably, we found a unique domain organization in a few species, including *Perkinsus marinus* and *Ostreococcus tauri*, in which succinate dehydrogenase (fumarate reductase) and cytochrome b5 domains are fused to the C-terminus of the Ero1 domain, resulting in a multi-domain protein of ~120 kDa (Fig. 2C, our unpublished observation). This organization suggests a novel link between the Ero1-based oxidative cascade and the respiratory chain, as is seen in mitochondrial Erv1/Mia40 or the bacterial disulfide bond protein A (DsbA)/B system (14, 30). It is thus conceivable that alternative electron flow cascades are active under anaerobic or hypoxia conditions *in vivo*.

Erv/ALR family members, which are mitochondrion-resident oxidases, are also highly conserved among eukaryotes, but ER-resident Erv2 members are encountered only in fungi. Conversely, QSOX/VKOR members are distributed more widely, except in fungi (and several protists). The ER-resident peroxidases, Prx4 and GPx7/8, exist only in the

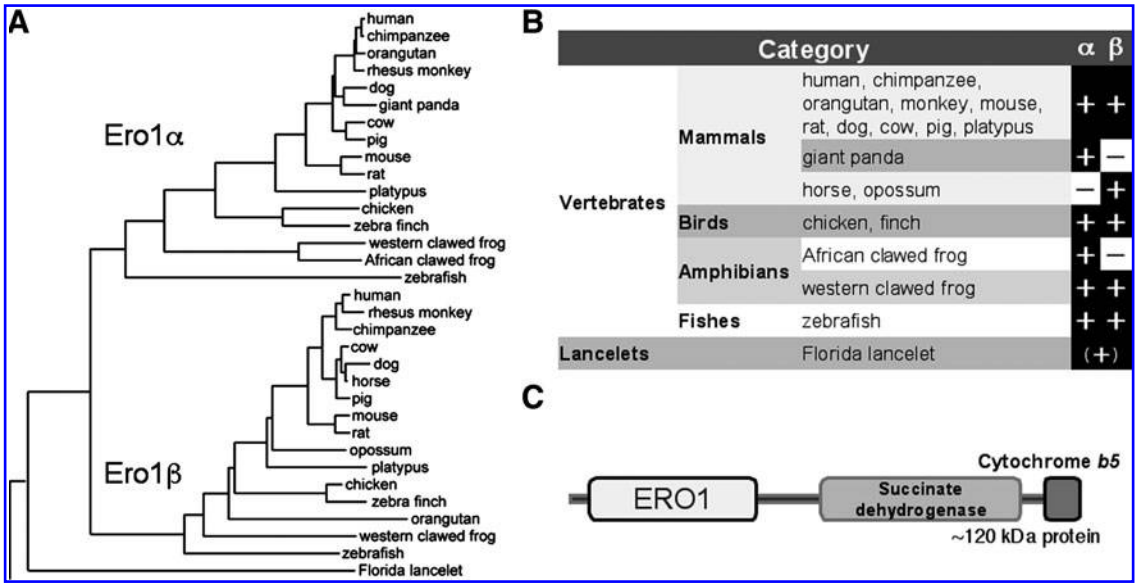

FIG. 2. Phylogenic perspectives of Ero1-family members. (A) Phylogenic tree at the branching point of Ero1α and Ero1β. Calculation of the phylogenic tree was performed by the neighbor-joining method, using the dataset of Ero1 family proteins (133 species shown in Supplementary Table S1) aligned by the MAFFT method (www.genome.jp/tools/mafft/; strategy option L-INS-i) (36). **(B)** Comparison of Ero1α and/or Ero1β coding vertebrates. The *Florida lancelet* codes an *Ero1* gene that cannot be classified as Ero1α or Ero1β. **(C)** Unique domain organization of Ero1-coding gene from *Perkinsus marinus* ATCC 50983, *Ostreococcus tauri*, *Micromonas* sp. RCC299, and *Micromonas pusilla* CCMP1545, in which Ero1 protein is fused with succinate dehydrogenase (fumarate reductase) and cytochrome b5 domains. Ero1, endoplasmic reticulum oxidoreductin 1.

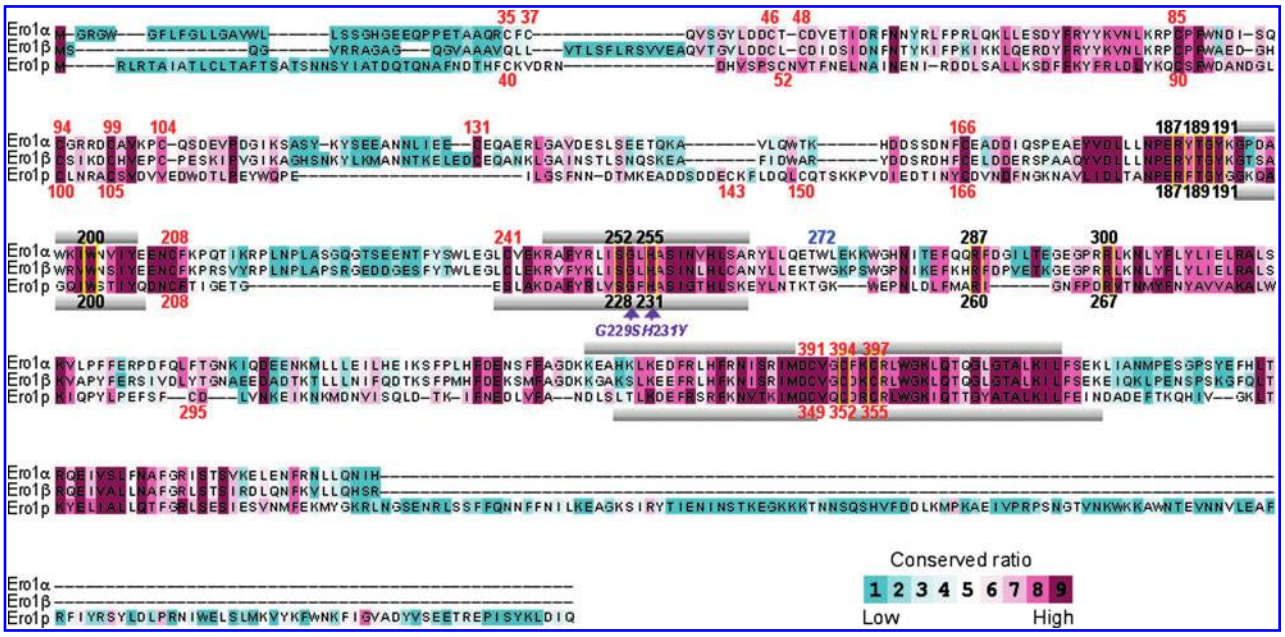

FIG. 3. Multiple alignment of Ero1α/β/p. The conserved ratio was analyzed by the ConSurf server (http://consurf.tau.ac.il/) (6) using alignment data of Ero1 family proteins (see also Fig. 2) and classified by color; red to blue color corresponds to high to low conservation, respectively. The four-core α-helices of Ero1α and Ero1p are indicated by gray boxes above and below the sequences. Red numbers indicate cysteines, and FAD-binding amino acids are highlighted by yellow boxes. (To see this illustration in color the reader is referred to the web version of this article at www.liebertonline.com/ars). FAD, flavin adenine dinucleotide.

animal kingdom after branching from nematodes (see Supplementary Table S1).

Structural Overview of Disulfide-Generating Catalysts

Recent in-depth studies reveal structural and mechanistic similarities and differences among the enzymes dedicated to *de novo* protein disulfide generation. To date, crystal structures are solved for the FAD-dependent soluble oxidases Ero1 (23, 31), Erv1/ALR (54, 58), Erv2 (25), and QSOX (only the C-terminal half) (1); quinone-dependent membrane disulfide oxidoreducatses, *Escherichia coli* DsbB (32, 33, 59) and a bacterial homolog of VKOR (40); and the cofactor-independent peroxidases Prx4 (57) and GPx7/8 (43) (Table 1). These crystal structures revealed that both quinone- and FAD-dependent disulfide-generating enzymes are composed of two functional regions—a flexible loop (also called a shuttle or distal loop) that contains a shuttle disulfide and a four-helix bundle core region that binds cofactors adjacent to an active-site dithiol/disulfide motif (Cys-X-X-Cys) (Fig. 4A). While the position and context of the shuttle disulfide in the flexible loop differs among the oxidases, the general mechanistic view is that the flexible loop accepts electrons from the substrate(s), usually thioredoxin-related proteins such as PDI or DsbA, followed by the transfer of electrons to the cofactor through the active-site Cys-X-X-Cys motif. Substrate-induced conformational rearrangement in the flexible loop likely plays a critical role in unidirectional electron transfer in these oxidative pathways, as has been demonstrated most clearly in *E. coli* DsbB-DsbA (32, 33). This loop may also protect the active-site motif, hampering direct and deregulated oxidation of nonspecific substrates (29, 30, 47).

Whereas Ero1-family enzymes assume a single globular fold rich in α-helices (23, 31), the Erv-family proteins form non-covalent/covalent homodimers (Fig. 4B) (19, 21). Each protomer has two functional regions: the FAD-binding core region that contains a redox-active Cys-X-X-Cys motif near the isoalloxazine ring of FAD, and a flexible shuttle arm with a di-cysteine motif outside the core region. Although the location of this di-cysteine motif varies among species (N-terminally in yeast Erv1p and human ALR, and C-terminally in Arabidopsis Erv1 and yeast Erv2p), it commonly mediates intermolecular electron transfer from the substrate to the catalytic core region of the protomer. As is the case for Ero1, the flexible arm defines the specific substrates and prevents nonspecific oxidation.

The crystal structure is also available for a bacterial homolog of VKOR in complex with its naturally fused thioredoxin-like domain. This structure revealed that the enzyme has remarkable structural and mechanistic similarities to the *E. coli* DsbB-DsbA complex (Fig. 4B) (40). The catalytic core of VKOR forms a four-helix bundle that embraces a quinone species. VKOR also has four conserved catalytic cysteines, two of which are active-site cysteines in a Cys-X-X-Cys motif while the other two are shuttle cysteines in an extramembrane loop. A genomic gene search suggested a functional role for VKOR in protein disulfide formation; some bacteria that code DsbA but lack DsbB possess VKOR, presumably as a substitute for DsbB (18).

Feedback Regulation of Ero1α/β/p

As addressed above, Ero1-family proteins undergo feedback regulation to prevent hyperoxidation of the ER. It is now known that yeast and human Ero1s have similar, but not identical, regulatory mechanisms (Fig. 5).

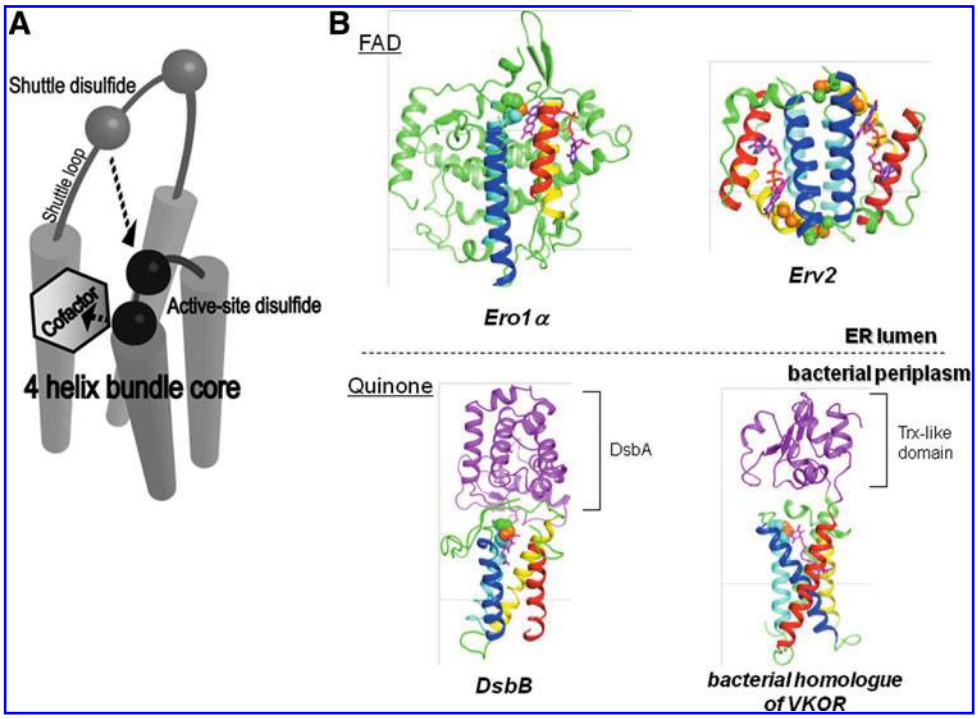

FIG. 4. Converged structural architecture found in disulfide-generating enzymes. (A) A simplified schematic illustration of the common structure and electron flow mechanism in disulfide-generating enzymes. Four spheres indicate the catalytically essential cysteines, two of which form shuttle disulfides (light gray) on the flexible loop while the other two form active-site disulfides (dark gray) located proximal to the cofactor (quinone or FAD). *Dashed arrows* indicate the electron transfer pathway. **(B)** Overview of FAD-dependent soluble oxidases (Ero1α and Erv2), quinone-dependent membrane oxidases (DsbB in complex with DsbA), and bacterial VKOR in complex with its naturally fused thioredoxin-like domain. The four helices that constitute the cofactor-embracing bundle are shown in blue, cyan, red, and yellow for clarity. Bound FAD and ubiquinone are represented by sticks, and catalytic cysteines are indicated by spheres (sulfur atom in orange). (To see this illustration in color the reader is referred to the web version of this article at www.liebertonline.com/ars).

Yeast Ero1p and Human Ero1α/β

Yeast Ero1p has two noncatalytic/regulatory cysteine pairs, Cys90-Cys349 and Cys150-Cys295 (49). While the crystal structures of the regulated and deregulated forms of Ero1p revealed no conformational changes within 25 Å of the active site (28), disulfide bond formation between the regulatory cysteines strictly regulates PDI oxidation of Ero1p, probably due to the restricted motion of the loop containing the electron shuttle disulfide (Cys100-Cys105). During

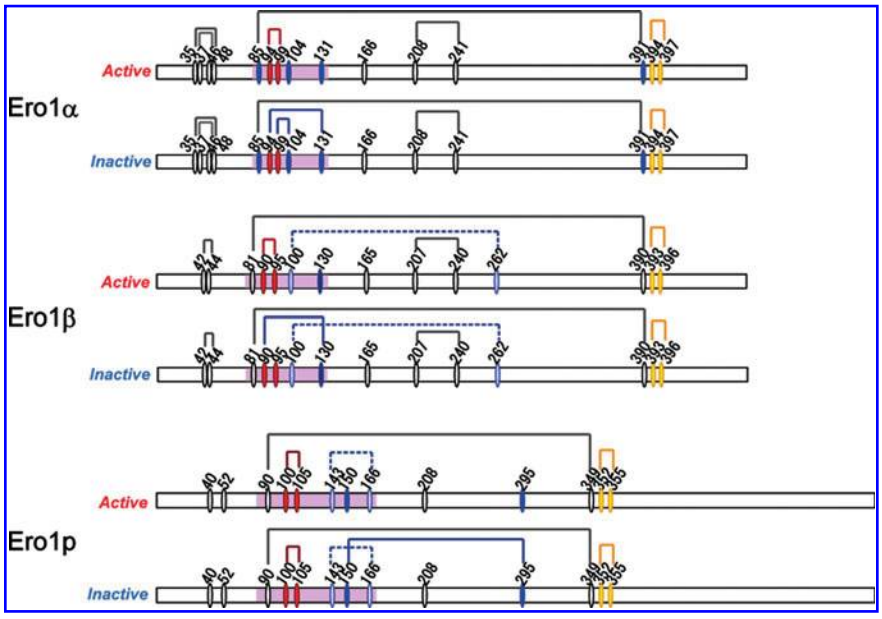

FIG. 5. Disulfide connectivity in human Ero1α and Ero1β and yeast Ero1p. Solid blue, dashed blue, yellow, and gray lines indicate the regulatory disulfide, auxiliary regulating disulfide, active-site disulfide, and structural disulfide, respectively. Circles represent cysteine residues (numbered as indicated), whose color corresponds to those of the lines. The flexible loop region containing a shuttle disulfide is shown in pink. Note that the disulfide connectivity in Ero1β is based on biochemical analyses (56) and lacks structural evidence. (To see this illustration in color the reader is referred to the web version of this article at www.liebertonline.com/ars).

activation of Ero1p, the Cys143-Cys166 disulfide is the first disulfide to be reduced, and concomitant reduction of Cys150-Cys295 is the critical step for its full activation (28).

In human Ero1α, all the regulatory cysteines (Cys94, Cys99, Cys104, and Cys131) are concentrated in a single loop region (31). Unlike the regulatory cysteines of yeast Ero1p, two human regulatory cysteines, Cys94 and Cys99, also act as catalytic cysteines in the active form (Fig. 5). That is, the active form of Ero1α contains a shuttle disulfide between Cys94 and Cys99, while Cys104 and Cys131 are reduced. On the other hand, the inactive form possesses Cys94-Cys131 and Cys99-Cys104 disulfide bonds (3, 7). Crystal structures of the constitutively active and inactive mutants of Ero1α demonstrated that these regulatory cysteines are positioned in an intrinsically flexible loop whose electron shuttle ability is finely tuned by the intra-loop disulfide rearrangement (31). Moreover, the active form with a flexible shuttle loop had a significantly higher affinity for PDI than the inactive form with a more constrained loop (42).

Ero1β seems to exert another type of regulatory mechanism (16, 56) in which the Cys90-Cys130 disulfide bond functions as the main regulatory switch to modulate activity, and Cys100-Cys262 plays an accessory role in its regulation. While the relatively high sequence homology of Ero1α and Ero1β (~65%) suggests a similar structure and mechanism of operation (Fig. 3), the latter is regulated more loosely than the former (56).

For long-range disulfide bonds, that is, Cys90-Cys384 (Ero1p), Cys85-Cys391 (Ero1α), or Cys81-Cys390 (Ero1β), reduction is not always necessary for full activation of any Ero1-family protein (4, 28, 49, 56). Thus, the long-range disulfide bond is likely to be important for the structural stability or proper oxidative activity of Ero1-family enzymes rather than for their negative regulation.

FAD-Binding Mode

To exert oxidase activity, Ero1-family enzymes utilize noncovalently bound FAD, which readily transfers electrons to molecular oxygen, generating H_2O_2 (ROS) as a byproduct. FAD consists of an isoalloxazine ring, a di-phosphate group and an adenosine monophosphate (AMP) unit. The FAD moiety can assume several conformations (extended, bent, and contact) depending on the type of flavoenzyme (Fig. 6A). In the extended conformation, the isoalloxazine and adenine rings are stretched out by more than 20 Å, whereas the contact conformation places these two rings in close contact (21). The FAD in Ero1 assumes a bent conformation, an intermediate between extended and contact, in which the isoalloxazine ring located at the center of the four-helix bundle and the adenine ring exposed on the protein surface are connected by a bent di-phosphate group (Fig. 6B). These two rings are separated by ~12 Å between the isoalloxazine C8 methyl group and the

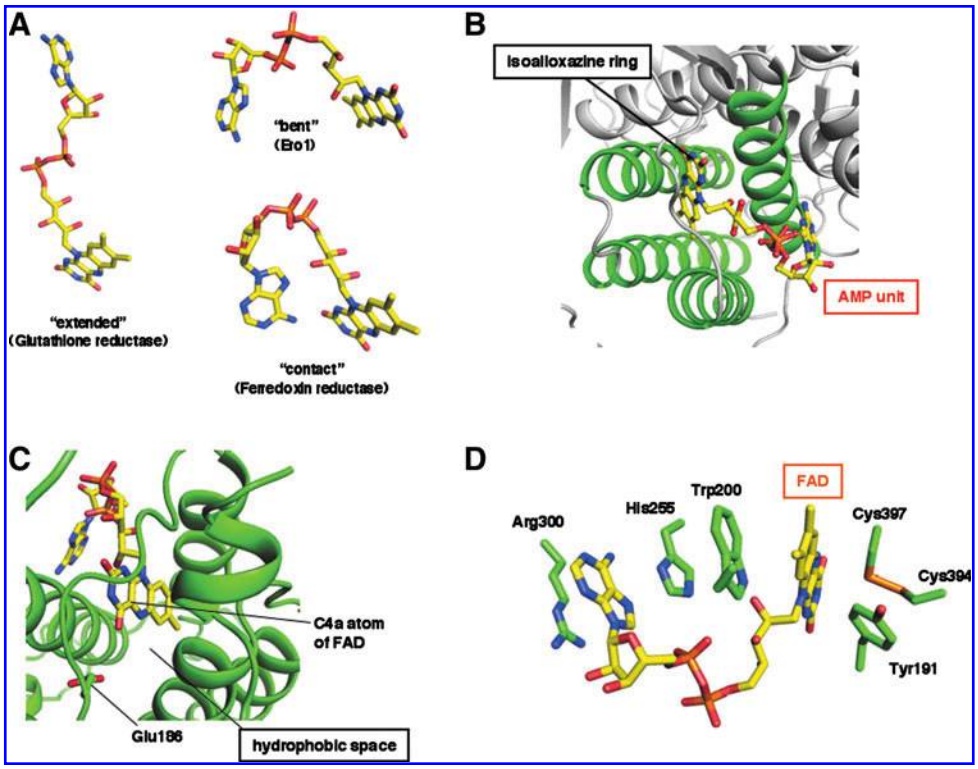

FIG. 6. FAD-conformations and FAD-proximal amino-acids. (A) FAD assumes an extended conformation in glutathione reductase (PDB ID; 1GRB), a bent conformation in Ero1α (3AHQ), and a contact conformation in ferredoxin reductase (1A8P), respectively. **(B)** FAD and its neighboring structure in human Ero1α. The FAD isoalloxazine ring (yellow stick) is entirely embedded within the four-helix bundle of the protein (green ribbon) and is shielded by its own di-nucleotide moiety, whereas the FAD AMP unit is exposed on the protein surface. **(C)** Local structure near the isoalloxazine ring of FAD in human Ero1α. **(D)** FAD-binding mode in human Ero1α. Residues involved in aromatic ring stacking with the FAD moiety are numbered and shown by stick representation. (To see this illustration in color the reader is referred to the web version of this article at www.liebertonline.com/ars). AMP, adenosine monophosphate; PDB, protein database.

adenine N7 nitrogen and sandwich two amino acid side chains (W200 and H255) through virtually parallel stacking (Fig. 6C). In this geometry, the flavin C4a atom is remote from the nearest cysteine Cys397 by only 3.3 Å, within range for a Cys-FAD charge transfer complex and adduct after reduction of the Cys394-Cys397 active-site disulfide. The residues involved in FAD binding are highly conserved (Fig. 3), and mutations of these residues grossly impaired its stability or activity, as exemplified by the two conditional phenotypes of yeast Ero1p: a temperature-sensitive mutation, *ero1-1* (G229S), and a dithiothreitol-hypersensitive mutant, *ero1-2* (H231Y) (22, 23, 45). Equivalent mutations in Ero1β made this enzyme susceptible to misoxidation and aggregation, particularly during temperature or redox stress, suggesting the importance of the FAD-binding structure in conformational stability and proper functioning of Ero1-family enzymes (16).

Despite the low sequence similarity between Erv- and Ero1-family proteins, striking structural similarities are observed, especially in the FAD-binding mode. Both Erv1/ALR and Erv2 have a scaffold comprised of five α-helices, four of which form a bundle that accommodates the isoalloxazine ring of FAD (19). It should be noted that some remarkable differences lie within the entrance gate for molecular oxygen (see also the next section). The Erv1/2 structure revealed a short hydrophobic channel through which O_2 seems to readily approach the isoalloxazine ring (19, 21). Moreover, Erv-family proteins lack noncatalytic regulatory cysteines in contrast to the Ero1-family. Erv1 activity, instead, depends on the cellular oxygen concentration (10), being compatible with a molecular switch model that adjusts mitochondrial activity to the oxygen levels in the cell. However, cytochrome c is also a likely physiological oxidant of Erv1/ALR enzymes (20). This alternative pathway passes electrons *via* cytochrome c oxidase to molecular oxygen to produce water, thus avoiding the generation of H_2O_2 in the mitochondrial intermembrane space. This explains the previous observation that while the K_m for O_2 for ALR is $\sim$240 μM, the intramitochondrial O_2 concentration in a respiratory tissue is just 5 μM (20).

Oxygen Pathways

Although *in vitro* studies demonstrated that molecular oxygen is used as the terminal electron acceptor of Ero1 (24, 56), the crystal structures of Ero1 proteins did not reveal the pathway or channel for entry of an O_2; the isoalloxazine ring is buried inside the molecule, seemingly shielded from access of molecular oxygen (Fig. 6B). This fact suggests that if electrons are transferred from bound FAD to molecular oxygen, some dynamic motions need to occur in the surrounding area to facilitate oxygen entry. The previous theoretical approach suggested spontaneous protein-guided O_2 diffusion from the bulk solvent to preorganized protein cavities in several flavoprotein monooxygenases and oxidases (8). Multiple pathways that guide oxygen diffusion to the reacting C4a atom of the flavin cofactor are also likely to occur in Ero1-family enzymes. In line with this, Ero1α has a relatively hydrophobic surface patch close to the isoalloxazine ring (Fig. 7B), which may act to capture O_2 molecules from the bulk solvent. Moreover, there is a hydrophobic space near the C4a flavin atom of Ero1α, which would contribute to accommodating O_2 and facilitating formation of the C4a-hydroperoxide intermediate, possibly with the aid of acidic Glu186 (Fig. 6C).

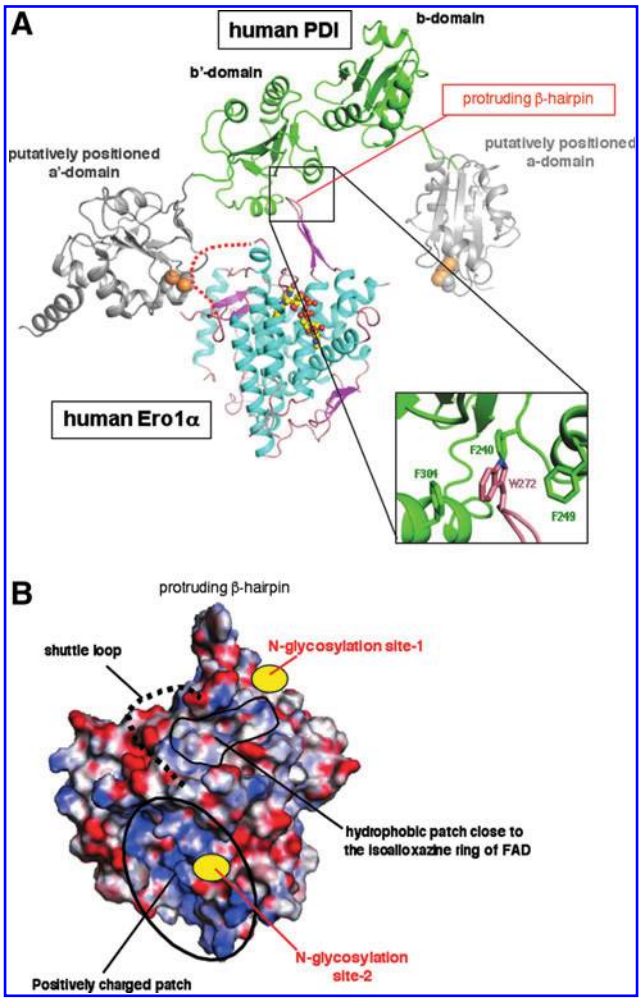

FIG. 7. Specific and functional interaction of Ero1α-PDI. **(A)** The predicted Ero1α-PDI complex model (42). The *inset* highlights the interface of these two enzymes, in which Trp272 from Ero1α and its neighboring Phe240, Phe249, and Phe304 from PDI closely contact each other. The FAD molecule in Ero1α is shown by small yellow-orange spheres. The redox-active sites of PDI are shown by larger orange spheres. Note that the redox-active site in the PDI a'-domain is predicted to reside close to the shuttle loop of Ero1α (red dotted line). **(B)** Electrostatic surface representation of human Ero1α (31). The orientation of Ero1α is the same as in Figure 4B. The shuttle loop is indicated by a dotted line. The positively charged patch and the relatively hydrophobic surface region near the isoalloxazine ring of FAD are encircled, respectively. Regions of basic potential are in blue ($>$20 kBT/e) and acidic regions are in red ($<-$20 kBT/e). Yellow ovals indicate the positions of two glycosylation sites. (To see this illustration in color the reader is referred to the web version of this article at www.liebertonline.com/ars). PDI, protein disulfide isomerase.

Detailed inspection of Ero1 structures may allow us to propose an even more radical hypothesis regarding an alternative O_2 reaction pathway. As addressed above, the isoalloxazine ring, side chains of Trp200 and His255, and the adenine ring are aligned nearly parallel with each other in this order, suggestive of their resonance structure (Fig. 6D). This feature is also observed in Erv/QSOX family proteins. Taking

into account the fact that only the AMP unit of bound FAD is exposed on the Ero1 surface (Fig. 6B), the adenine ring might make a direct redox interaction with molecular oxygen or other unknown electron acceptors under anaerobic conditions. To characterize the exact electron transfer pathway(s) between Ero1 and molecular oxygen, further biochemical and computational studies are required (8, 34).

Catalytic and Specific Association with PDI

Among the ER-resident oxidoreductases examined so far, PDI is the preferred substrate of Ero1 (31, 56). Human and yeast PDI, hereafter named hPDI and Pdi1p, respectively, consists of two redox-active domains (a and a′) with a catalytic Cys-Gly-His-Cys (CGHC) motif and two noncatalytic b and b′ domains, which are lined up in the order a-b-b′-a′ with an α-helical extension at the C-terminus (26). Importantly, the cellular activity of Ero1α/Ero1p is most likely controlled by the availability of reduced hPDI/Pdi1p (2, 48), which presumably senses the redox state in the ER and reduces/rearranges the regulatory disulfide bonds of Ero1 enzymes.

The binding modes of Ero1-PDI seem to be different in yeast and humans. Human Ero1α/β oxidizes the C-terminal a′-domain of hPDI much more effectively than the N-terminal a-domain (7, 13, 42), while the intra-molecular electron relay from the a- to the a′-domain within hPDI is possible during Ero1α-catalysis of hPDI oxidation (4). In contrast to human Ero1α, Ero1p preferentially oxidizes the a-domain of Pdi1p (4, 13, 55). Recent studies clearly demonstrate that a protruding β-hairpin of Ero1α, in which Trp272 appears to be most critical, interacts with the hydrophobic pocket in the hPDI b′-domain, leading to effective oxidation of the a′-domain (42) (Fig. 7A). This view was strongly corroborated by a protein engineering approach, in which swapping of the b′-domain between PDI and ERp57, another ER-resident oxidoreductase with a PDI-like domain organization but a low reactivity with Ero1α, substantially altered their affinity and reactivity for Ero1α (31). In addition to such hydrophobic interactions, electrostatic interaction likely contributes to their functional association. The overall surface of PDI is highly acidic, while Ero1α contains a positively charged patch comprised of several basic residues (Arg83, Arg383, and Arg387) near the shuttle loop (Fig. 7B). Mutation of these basic residues compromised the PDI oxidation activity of Ero1α, suggesting an auxiliary role for electrostatic interaction in functional Ero1α-hPDI interplay (31). There are also two well-conserved glycosylation sites in Ero1α (50): Asn280, located at the base of the protruding β-hairpin (N-glycosylation site-1), and Asn384, near the positively charged patch (N-glycosylation site-2) (Fig. 7B). It remains to be clarified whether glycosylation affects not only the stability/solubility of Ero1α but also its affinity/reactivity for PDI.

In either human or yeast, the most important question is how Ero1 activity is regulated in living cells. It is known that Ero1α rescues the *ero1-1* yeast temperature-sensitive mutant (9), despite the different binding modes in yeast and human Ero1-PDI. Perhaps Ero1α and Ero1p are activated by a similar ER redox environment, including the availability of PDI, allowing Ero1α to function as an alternate PDI oxidase in the yeast ER. A more critical issue to be elucidated is the inactivation step of Ero1s, specifically, whether this step proceeds by itself or is mediated by other factors.

Future Perspectives

Since the recent observation of only modest phenotypes in $Ero1\alpha^{i/i};\beta^{i/i}$ mice, much attention has been paid to the plausible existence of multiple oxidative pathways in mammalian cells. During evolution into a higher order of living beings, ER-resident disulfide-generating enzymes have diversified, probably to cope with the wide variety of substrate proteins and redox phenomena that occur in the ER. We speculate that multiple oxidative protein folding pathways serve as a failsafe network to maintain ER protein homeostasis even under various stresses or genetically deficient conditions. The next critical issue will be to describe extensively and accurately how each oxidative pathway works distinctly and redundantly in living cells.

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Abbreviations Used

Å = angstrom
AERO = *Arabidopsis thaliana* endoplasmic reticulum oxidoreduclin1
ALR = augmenter of liver regeneration
AMP = adenosine monophosphate
CHOP = C/EBP homologous protein, also known as growth arrest- and DNA damage-inducible gene 153 (GADD153)
Dsb = disulfide bond protein
DTT = dithiothreitol
ER = endoplasmic reticulum
Ero1 = endoplasmic reticulum oxidoreductin 1
Erv1/2 = proteins originally termed essential for respiration and vegetative growth
FAD = flavin adenine dinucleotide
GPx = glutathione peroxidase
GSH = reduced glutathione
GSSG = oxidized glutathione
H_2O_2 = hydrogen peroxide
HIF-1 = hypoxia-inducible factor 1
Mia = mitochondrial intermembrane space import and assembly
O_2 = oxygen molecule
PDB = protein database
PDI = protein disulfide isomerase
PDX1 = pancreatic transcription factor
Prx = peroxiredoxin
QSOX = quiescin sulfhydryl oxidases
ROS = reactive oxygen species
VKOR = vitamin K 2,3-epoxide oxidoreductase

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