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FORUM REVIEW ARTICLE

Erv2 and Quiescin Sulfhydryl Oxidases: Erv-Domain Enzymes Associated with the Secretory Pathway

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Abstract

Significance: Members of the Erv/ALR/QSOX protein family contain an Erv sequence module and catalyze protein disulfide bond formation. Erv enzymes impact protein function within and outside cells that affects both normal and malignant cell growth. This protein family is named for its founding members: Erv1 (essential for respiratory and vegetative growth 1) and ALR (augmenter of liver regeneration), homologous mitochondrial proteins from yeast and mammals, respectively, and QSOX (quiescin sulfhydryl oxidase), an oxidase secreted from quiescent cells. This review will focus on a subset of Erv proteins that are localized within the secretory pathway: Erv2-like proteins, proteins present in the endoplasmic reticulum of fungi, and QSOX proteins, proteins localized within the secretory pathway and extracellular space and present in most eukaryotes, but not fungi. ***Recent Advances:*** A wealth of structural and biochemical data has been obtained for Erv2 and QSOX proteins. These data have identified a generally conserved catalytic mechanism and structure for the Erv2 and QSOX proteins with unique features for each enzyme. ***Critical Issues:*** Many fundamental questions remain about the activity for these proteins in living cells including the partners, pathways, and locations utilized by these enzymes *in vivo*. ***Future Directions:*** A more comprehensive understanding of the cellular roles for Erv2 and QSOX enzymes will require identification of their partners and substrates. Also, determining when Erv2 and QSOX function during growth and development, and how changes in levels of active Erv2 and QSOX impact cell function, is necessary to facilitate a better understanding of these intriguing enzymes. *Antioxid. Redox Signal*. 16, 800–808.

Introduction

An Erv domain (alternately referred to as an Erv/ALR domain) is a protein module of ~80–100 amino acids of sequence homology. Proteins with an Erv domain have sulfhydryl oxidase activity and can oxidize a variety of thiol-containing compounds *in vitro*, including small molecules and proteins. In cells, Erv proteins have been shown to facilitate oxidation of protein cysteines to form disulfide bonds (7, 40, 48).

Catalytic activity of Erv enzymes relies on an active site formed by a conserved Cys-X_2-Cys motif (where X is any amino acid other than cysteine) and a noncovalent, bound flavin adenine dinucleotide (FAD) cofactor. Erv proteins transfer disulfide bonds to thiol-compounds *via* thiol-disulfide exchange (Fig. 1), wherein two substrate thiols are oxidized to a disulfide and, in exchange, a disulfide between the Erv Cys-X_2-Cys pair is reduced to thiols. FAD facilitates reoxidation of the Erv Cys-X_2-Cys pair by mediating the transfer of electrons received by the active-site cysteines during substrate oxidation to nonthiol electron acceptors. *In vitro*, oxygen can accept electrons directly from the FAD cofactor, and this transfer reaction yields hydrogen peroxide (30, 48, 58, 59). Transfer of electrons to oxygen and/or production of peroxide by Erv proteins in cells remain to be demonstrated. Family members that transfer electrons to cytochromes, both *in vivo* and *in vitro*, also have been described (6, 13, 17).

Characterized Erv proteins utilize partner oxidoreductases to mediate the transfer of disulfides generated by Erv proteins to a range of polypeptides in cells (40, 47, 48). The pathway used by Erv enzymes to oxidize protein substrates follows a general design that is considered characteristic for many cellular pathways for protein oxidation (for recent review see Ref. 16) (Fig. 2). At a basic level, these pathways involve three steps: (i) *de novo* disulfide formation by an enzyme that uses small-molecule redox chemistry (*e.g.*, oxygen and FAD) to generate a disulfide bond between an active-site Cys-X_2-Cys pair, (ii) transfer of the disulfide bond to an active-site pair in an oxidoreductase that serves to carry the disulfide from the generator to the substrate, and (iii) oxidation of substrate cysteine thiols to a disulfide bond by thiol-disulfide exchange between the disulfide carrier oxidoreductase and substrate (Fig. 2). An enhanced catalytic efficiency has been observed for an Erv protein with its partner oxidoreductase, relative to a

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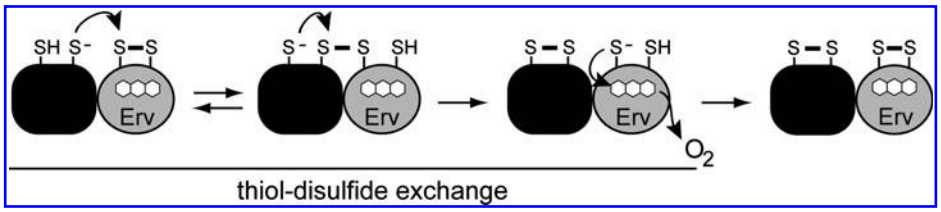
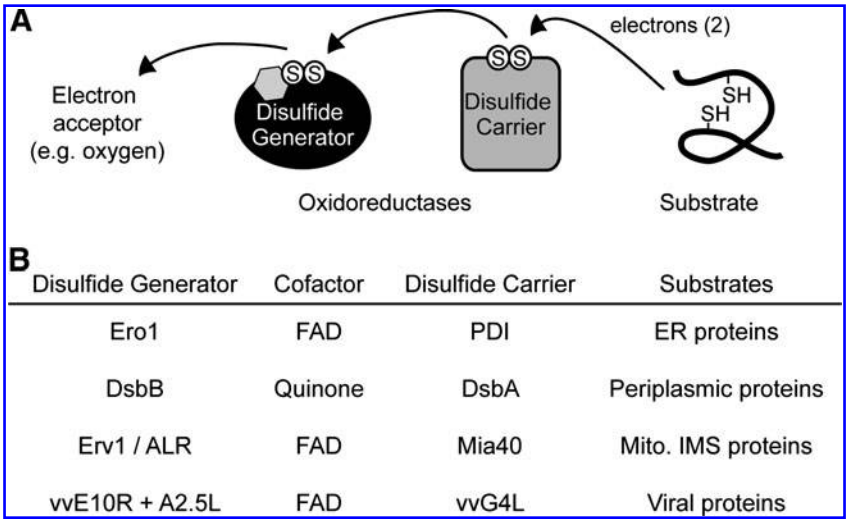


FIG. 1. Thiol-disulfide exchange reaction. In an exchange reaction, a thiolate anion, formed by the deprotonation of a free thiol, displaces one sulfur of a disulfide bond in an oxidized species (*e.g.*, an oxidized Erv active-site pair). This process leads to the formation of a transient mixed-disulfide bond connecting the originally oxidized and reduced cysteine pairs. The mixed-disulfide intermediate is resolved by attack of the mixed-disulfide bond by a thiolate anion derived from the remaining thiol in the originally reduced cysteine pair. The net result of the thiol-disulfide exchange process is the oxidation of the originally reduced species and the concomitant reduction of the initially oxidized species. A reduced Erv active site is reoxidized for another cycle through transfer of electrons to a flavin cofactor and molecular oxygen. Note for QSOX, the exchange reaction likely involves intramolecular thiol-disulfide exchange between the active site and Trx domains in a single QSOX protein. For Erv2, the reaction includes intermolecular thiol-disulfide exchange between the active site and secondary pair of cysteines in associated Erv2 protomers (see text for more details). Erv, essential for respiratory and vegetative growth; PDI, protein disulfide isomerase.

model unfolded polypeptide (14). These data suggest preferred pathways exist within cells for electron flow to the Erv module. However, it is worth noting that an Erv enzyme can facilitate protein oxidation in the absence of its physiological partner. For example, expression of a mitochondrial Erv enzyme in the cytoplasm of bacteria (in the absence of its mitochondrial partner) can promote oxidation of alkaline phosphatase and phytase (25). In this particular case, a substantial level of Erv protein relative to substrate (coexpression of Erv and substrate from a polycistronic vector) likely facilitates polypeptide oxidation. Alternatively, the Erv enzyme may coopt a bacterial enzyme to mediate electron transfer.

A consensus sequence signature for the Erv domain in cellular proteins has emerged as Gly-X_3-Trp-X_3-His-X_5-Phe/Tyr-X_{23}-Pro-Cys-X_2-Cys-X_N-His-Asn-X_2-Asn (where X_N denotes a variable number of amino acids; active-site cysteines are underlined) (18). Viral proteins with an Erv module diverge slightly from the canonical eukaryotic motif and lack some of the residues in the cellular Erv signature, following a rough consensus of Trp-X_3-His/Phe-$X_{37–41}$Cys-X_2-Cys-X_2-His/Gly (36). Most of the invariant residues in the Erv signature motif interact with or accommodate the FAD cofactor. Many Erv domain–containing proteins also include cysteines in a Cys-$X_{1–4}$-Cys arrangement either N- or C-terminal to the Erv module. The presence of additional cysteine pairs was originally anticipated to be a general feature for the Erv module, and for a subset of Erv domain–containing proteins these cysteines are required for catalytic activity (14, 20, 28, 58). However, viral Erv domains are not flanked by a Cys-$X_{1–4}$-Cys sequence (21), and not all proteins with an Erv



B

Disulfide Generator	Cofactor	Disulfide Carrier	Substrates
Ero1	FAD	PDI	ER proteins
DsbB	Quinone	DsbA	Periplasmic proteins
Erv1 / ALR	FAD	Mia40	Mito. IMS proteins
vvE10R + A2.5L	FAD	vvG4L	Viral proteins

FIG. 2. Generalized pathway for protein oxidation by disulfide relay between proteins. (A) Disulfide formation between cysteines in a substrate protein is facilitated by thiol-disulfide exchange with a thiol-oxidoreductase, referred to here as a disulfide carrier. The active-site cysteines in the carrier are maintained in an active oxidized form through thiol-disulfide exchange with second oxidoreductase (a disulfide generator). The generator ultilizes a small-molecule cofactor to mediate electron transfer between its cysteines and an electron acceptor (*e.g.*, oxygen). Transfer of electrons to a small molecule by the generator facilitates formation of a disulfide bond within the disulfide generator enzyme itself. *Arrows* denote transfer of two electrons. **(B)** Examples of characterized cellular pathways that use a disulfide generator and carrier as described in **(A)**. Reviewed by Depuydt *et al.* (16) and Hakim and Fass (21). ER, endoplasmic reticulum; FAD, flavin adenine dinucleotide; IMS, inner membrane space.

domain require a distal cysteine pair for catalytic function (27, 35). Correspondingly, the presence of a second cysteine pair is not part of the Erv domain consensus.

The Erv module adopts a five-helix fold: four helices (α1–4) bundle to coordinate the FAD cofactor and a short fifth helix (α5) packs against the bundle (Fig. 3). The active-site Cys-X_2-Cys motif localizes to a turn between helices α2 and α3 and is juxtaposed to the flavin isoalloxazine ring. In the majority of structures, the Erv domain is found as a dimer. For all cellular Erv proteins with solved structures, the dimer interface is formed between helices α1 and α2 and their symmetry mates (α1′ and α2′) (1, 15, 20, 58, 61). The packing of the symmetry-related helices positions the active-site cysteines on opposite faces of the Erv protomers, suggesting a functional independence for each active site. More recently, an intrachain pseudo-dimer has been described, where the α1/α2 helices in the Erv module dimerize with a helix-rich region (HRR) that adopts an Erv-like fold, but lacks a flavin cofactor and catalytic activity (1) (discussed further in QSOX: Domain Organization and Structure section).

Among the viral proteins, however, there is a surprising diversity in quaternary structure, highlighting the ability of viruses to evolve and push the boundaries for the Erv fold. Three distinct intermolecular dimer interfaces have been observed for viral Ervs, formed between helices α1/α2 (22), α2/α3 (22), or α3/α4 (23) and the corresponding symmetry-related helices. Remarkably divergent in quaternary structure from the cellular Ervs, the later α3/α4 intermolecular dimer interface, observed for a baculovirus oxidase, brings together two intramolecular Erv pseudo-dimers (23). Currently how the different quaternary structures impact Erv oxidase activity is not known.

The Erv module is found in several protein contexts. These are grouped most often into two general classes: (i) proteins that contain a single Erv module (Erv/ALR family) and (ii) proteins that contain an Erv domain fused with a thioredoxin (Trx)-like domain (QSOX family). Single-domain Erv proteins can be subgrouped also by the presence or absence of N-terminal targeting information: (i) proteins with a mitochondrial presequence (Erv1/ALR proteins) for localization in the mitochondrial inner membrane space (IMS), (ii) proteins with a signal sequence (Erv2/Erv2 homologs) for targeting to the endoplasmic reticulum (ER), and (iii) virally encoded proteins that lack a presequence or signal sequence and are present in the cytoplasm during viral infection.

The localization of Erv domain-containing proteins to a range of organelles enables protein oxidation pathways in multiple cellular locations (Fig. 4). As this Forum is focused on folding within the ER, this review will center on Erv domain–containing proteins that localize to or transit through the ER: Erv2-like proteins, fungal enzymes that have been demonstrated to augment disulfide formation in the ER, and quiescin sulfhydryl oxidases (QSOX), proteins distributed within the secretory pathway and extracellular space and thought to allow for protein oxidation within or outside the cell. For recent reviews focused on the mitochondrial and viral Erv proteins see works by Hakim and Fass (21) and Riemer *et al*. (45). This review will discuss first the catalytic activities and structural features for Erv2 and QSOX proteins. This discussion will be followed by an examination of our knowledge of the cellular locations and activities for the Erv2 and QSOX enzymes. Here we will highlight some of the fundamental questions that remain related to the roles for Erv2 and QSOX proteins within cells.

Erv2 and QSOX Proteins: A Preface

Erv2 is a relatively small 22 kD protein characterized first in *Saccharomyces cerevisiae*. Erv2 was identified initially based on its sequence homology with Erv1, an essential mitochondrial protein also in *S. cerevisiae* (50). However, unlike *ERV1*, *ERV2* is not an essential gene and the name essential for respiration and vegetative growth 2 is an unfortunate moniker. QSOX are multidomain enzymes that range in size from ~50–80 kD.

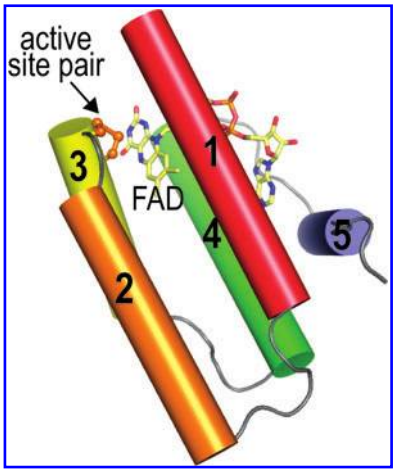


FIG. 3. Erv-fold structure. A single subunit of Erv2 is shown as representative of the Erv structural fold. Helices are represented as cylinders and numbered from amino to carboxy terminus. The active-site cysteines are shown as orange balls-and-sticks. The FAD is represented as sticks. (To see this illustration in color the reader is referred to the web version of this article at www.liebertonline.com/ars).

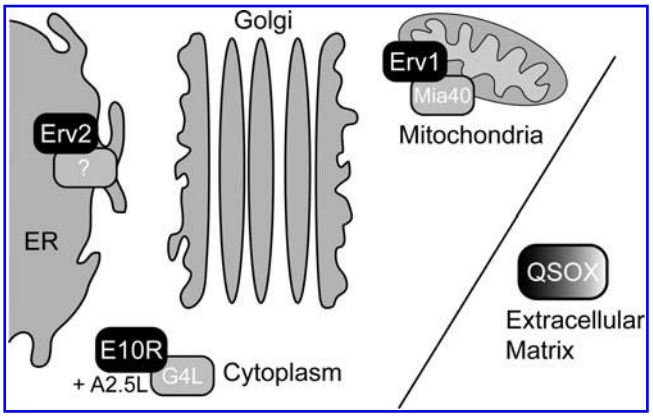


FIG. 4. Erv protein family members. A cartoon depicting the various cellular locations and partner enzymes described for characterized Erv proteins. For reviews of the viral (E10R/A2.5L-G4L) and mitochondrial (Erv1/ALR-Mia40) pathways see works by Hakim and Fass (21) and Riemer *et al*. (45). QSOX is depicted as extracellular, based on its initial description. However, QSOX has also been localized to internal organelles; refer to the main text for more details. ALR, augmenter of liver regeneration; QSOX, quiescin sulfhydryl oxidase.

QSOX proteins comprise an N-terminal redox-active Trx domain connected to a C-terminal Erv domain by a conserved helix-rich spacer. The Trx domain includes a conserved Cys-X_2-Cys motif, which is a hallmark of the thioredoxin-like superfamily of thiol-oxidoreductases.

Distribution of Erv2 and QSOX among genomes is non-overlapping. Erv2 orthologs (proteins with a signal sequence and an Erv consensus sequence) are present throughout the fungal kingdom. Sequences for Erv2-like proteins have not been observed in metazoans, plants, or protozoa. Conversely, QSOX proteins (proteins with a signal sequence and fused Trx and Erv domains) are widely distributed throughout the genomes of metazoa, plants, and protozoa and absent in fungal genomes. The localization of both the Erv2 and QSOX enzymes to the secretory pathway, together with the apparently distinct genomic distribution for these enzymes across species, suggests that the two families may be considered orthologous. However, Erv2 and QSOX enzymes are distinct in their structures, partner proteins, and likely localization. How these proteins evolved over time remains an open question.

Erv2: Domain Organization and Structure

Fungal genomes encode a single Erv2 ortholog. Erv2 proteins contain a single Erv domain flanked by the N-terminal signal sequence and a C-terminal extension, which contains an absolutely conserved second redox-active cysteine pair (Cys-Gly-Cys) (Fig. 5). The cysteines within the Cys-Gly-Cys sequence are required for *S. cerevisiae* Erv2 activity *in vivo* and mediate the transfer of electrons from substrate to the Erv2 active site (20). In the Erv2 structure, the Cys-Gly-Cys pair is found in a flexible domain that is close in space to the active site (20). Based on their function, the Cys-Gly-Cys cysteines are often referred to as "shuttle" cysteines. Use of a second redox-active pair, and the polypeptide context of the second pair, has been suggested to tailor the general oxidase activity provided by the Erv domain toward specific substrates (3, 28, 57, 58). Consistent with this idea, an Erv2 mutant that can bypass the shuttle mechanism shows activity toward a broader range of small molecules *in vitro* (57).

Erv2 was the first Erv family member crystallized. Its structure revealed the five-helix fold now characteristic of the Erv domain (described previously) (20) (Fig. 5). Within the Erv module of Erv2 is an additional pair of conserved cysteines separated by 16 residues (Cys-X_{16}-Cys pair). These cysteines form a disulfide bond that connects helix α5 to the helical bundle formed by helices α1–4 (20) (Fig. 5). This apparent structural Cys-X_{16}-Cys pair is present within other cellular single-domain Erv proteins, but is absent in both QSOX and the viral Erv enzymes. Mutagenesis of the Cys-X_{16}-Cys pair in Erv1 suggests that this cysteine pair is important to stabilize the overall protein fold (3, 28).

QSOX: Domain Organization and Structure

A single QSOX gene can be identified in the unicellular protozoa genomes of *Trypanosoma* and *Plasmodium*. In multicellular eukaryotes, several genes encoding QSOX proteins have been identified. *Homo sapiens* encode two QSOX genes (QSOX1 and QSOX2) (10, 60). Alternative splice forms of human QSOX1, referred to as QSOX1-L and QSOX1-S or alternatively as QSOX1a and QSOX1b, preserve or remove a C-terminal predicted transmembrane domain, respectively

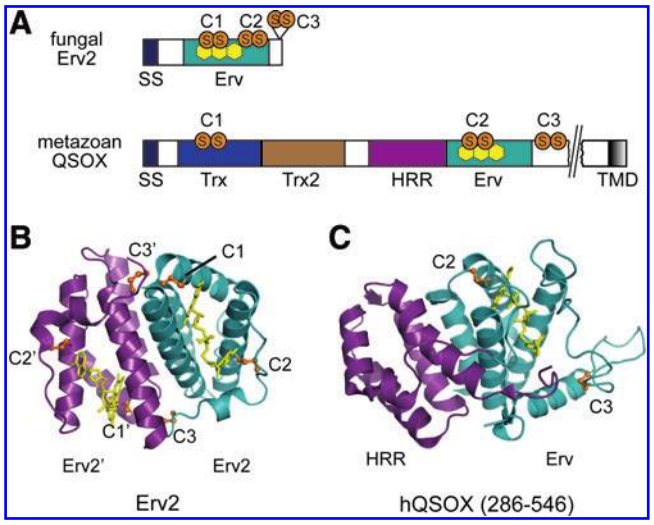


FIG. 5. Organization and structures for Erv2 and QSOX. **(A)** Cartoon representation of the domain organization for fungal Erv2 and metazoan QSOX. Conserved cysteines are noted as orange circles and numbered C1, C2, or C3 to correspond to the structure images in **(B)** and **(C)**. Boxes denote polypeptide sequence domains and yellow hexahedrons indicate regions that bind FAD. Metazoans encode multiple QSOX homologs that differ in the presence or absence of a C-terminal transmembrane domain, which is represented as a hatched line. **(B, C)** Cartoon representation for the structures of *Saccharomyces cerevisiae* Erv2 (20) and a fragment of human QSOX1 residues 286–546 (1). Erv2 is a homodimer. The two Erv2 protomers are labeled Erv2 and Erv2'. The Erv domain and upstream HRR in a single polypeptide chain of human QSOX adopt a fold similar to the Erv2 dimer, referred to as a pseudo-dimer. Cysteines conserved between orthologs are shown as orange balls-and-sticks and numbered according to **(A)**. FAD is represented as yellow sticks. (To see this illustration in color the reader is referred to the web version of this article at www.liebertonline.com/ars). SS, signal sequence; Erv, Erv domain; Trx, thioredoxin domain; Trx2, thioredoxin-like domain without an active site; HRR, helix-rich region; TMD, transmembrane domain; hQSOX, human QSOX.

(51). Additional QSOX gene duplications exist in other species. For example, four paralogs are found in *Drosophila* and three in *Caenorhabditis elegans*. The significance for multiple QSOX genes in a given organism is not clear. How the presence or absence of the C-terminal transmembrane domain in different splice forms impacts localization or function has also not been explored. Differences in cellular abundance and tissue distribution have been observed for mammalian QSOX1 and QSOX2 (12), suggesting that multiplicity may allow for differential regulation.

In all QSOX orthologs, immediately upstream of the Erv module is a HRR. Downstream to the Erv module is a highly conserved cysteine pair in a Cys-X_2-Cys arrangement (Fig. 5). Despite the expectation that these cysteines would act as an electron shuttle like observed for Erv2 (20), the C-terminal cysteines in QSOX are dispensable for catalytic activity *in vitro* (27, 35). Recent structural data for human QSOX1 support the absence of a catalytic role for the C-terminal Cys-X_2-Cys disulfide; in the X-ray structure, the Cys-X_2-Cys pair is physically separated in space from the active-site disulfide, on the opposite side of the protein

(1) (Fig. 5). It is interesting to consider why QSOX would not utilize a shuttle mechanism that is employed by cellular single-domain Erv proteins. If the shuttle mechanism confers specific interaction with a substrate partner, perhaps a specificity filter is not necessary for QSOX where its partner oxidoreductase (the Trx domain; discussed more in next section) is fused and maintained in close proximity to the Erv domain. Considering the significant conservation of these secondary cysteines between QSOX homologs, the absence of a clear catalytic or structural role for these cysteines is surprising. It remains possible that these cysteines play an important, but as yet uncharacterized, role in QSOX function.

The first high-resolution snapshot of QSOX comes from the recent structure of a fragment of human QSOX (1) (Fig. 5). The X-ray structure reveals that the Erv domain exists as an intrachain pseudo-dimer, formed between the Erv domain and the upstream HRR (1). The HRR domain adopts a compact four-helix bundle similar to the Erv fold, and the pseudo-dimer formed between the HRR and Erv modules shows striking similarity to the true dimer of Erv2 (1, 20) (Fig. 5). The HRR is not recognized by primary sequence as an Erv domain as it lacks both the Cys-X_2-Cys motif and the amino acids that would coordinate FAD (1).

A major distinction between the QSOX and Erv2 proteins is the presence of a redox-active Trx domain in QSOX following the signal sequence (Fig. 5). In metazoan QSOX proteins, two Trx domains are present in tandem (34). The second Trx domain (Trx2) lacks a Cys-X_2-Cys motif and is not redox active. QSOX with tandem Trx domains have been found only in metazoans. Plants and protists contain QSOX proteins with a single Trx module. The presence of noncatalytic Trx domains in non-Erv redox-active proteins has been proposed to provide a substrate-binding surface and/or aid in overall structural stability (26, 52). The purpose of the Trx2 domain in metazoan QSOX is unknown.

Catalytic Activity, Partners, and Substrates

Sulfhydryl oxidase activity for Erv2 and QSOX has been shown biochemically. The enzymatic activity of *S. cerevisiae* Erv2 was demonstrated first by the Lisowsky (19) and Kaiser groups (48). Both groups expressed and purified recombinant Erv2 and showed an ability for Erv2 to oxidize thiols to disulfides (19, 48). *S. cerevisiae* Erv2 is the only Erv2 ortholog characterized biochemically; in fact, ErvA from *Aspergillus niger* is the only Erv2 protein that has been characterized beyond its primary sequence (24). In contrast, QSOX proteins from several organisms have been studied both *in vitro* and *in vivo*. QSOX proteins are the best biochemically characterized of the Erv-containing proteins, and classical biochemical identification of QSOX as a sulfhydryl oxidase preceded identification of the Erv sequence motif [for details read Kodali and Thorpe (34)]. Many early kinetic studies utilized QSOX purified from chicken eggs; subsequent studies with *Homo sapiens*, *Bos taurus*, and *Trypanosoma brucei* QSOX show similar catalytic efficiencies and substrate specificities (35).

A direct comparison of the Erv module from QSOX (*sans* Trx domain activity) and Erv2 reveals similar catalytic activities. Small dithiol molecules can serve as substrates for Erv2, and Erv2 generates 63 disulfides per minute in the presence of 5 mM dithiothreitol (DTT) (48, 57, 59). An HRR-Erv truncation of *T. brucei* QSOX, or *T. brucei* QSOX with either Trx active-site cysteine replaced with serine (a Cys-X_2-Ser or Ser-X_2-Cys mutant), exhibits similar catalytic efficiency toward 5 mM DTT with turnover numbers in the range of 56–75 disulfides formed per minute (35). Erv2 exhibits limited catalytic efficiency (~2–3 disulfides formed per minute) toward reduced denatured proteins (57, 59). *T. brucei* QSOX Trx-domain active-site mutants also show poor activity toward a reduced polypeptide (~3 disulfides formed per minute), comparable to Erv2 activity (35). Similar Trx active-site mutants for human QSOX, with either Trx-domain active-site cysteine substituted with serine, show limited activity toward protein substrate (~20 disulfides formed per minute), which is a marginal increase in activity relative to the *T. brucei* mutants but still modest activity when compared with wild-type human QSOX (27).

The activity of full-length QSOX toward protein substrates is robust relative to Erv2. Avian QSOX catalyzes ~1000 disulfide bonds per minute into reduced denatured proteins (29). The increased activity of full-length QSOX relative to Erv2 suggests a synergy between the Trx and Erv domains in QSOX for disulfide formation in substrates. *In vitro* studies of QSOX proteins have confirmed a flow of oxidizing equivalents from the Erv domain to substrate proteins *via* the Trx-domain active-site cysteines (27, 35) (Fig. 6). Clearly, fusion of the Trx and Erv domains provides a catalytic advantage for oxidation of protein substrates relative to an isolated Erv domain, although a direct comparison of catalytic efficiency of QSOX relative to a complete Erv2 pathway for protein oxidation would require knowledge of the physiological oxidoreductase partner for Erv2.

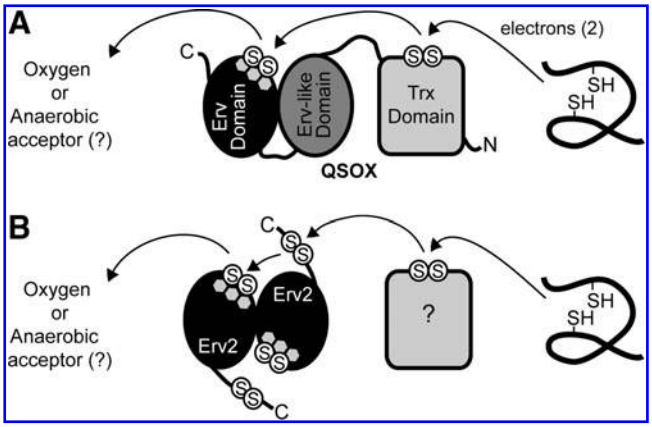


FIG. 6. Pathways for QSOX- or Erv2-mediated protein oxidation. (A) Electron transfer within and between cysteines in substrate, QSOX, and oxygen/alternate electron acceptors. Each two-electron transfer step is indicated with an *arrow*. Whether oxygen is an obligate acceptor for QSOX, or if QSOX can utilize alternate electron acceptors under anaerobic conditions is not known. **(B)** Proposed pathway for Erv2-mediated electron transfer from cysteines in substrate to an ultimate electron acceptor. Erv2 functions in cells grown anaerobically, suggesting an ability to transfer electrons to a nonoxygen electron acceptor (55). An unidentified thiol-oxidoreductase is thought to facilitate oxidation of substrates by Erv2, as Erv2 itself is a poor direct oxidant of protein thiols *in vitro*.

As Erv2 is a poor direct oxidant of polypeptide chains *in vitro*, it is anticipated that Erv2 partners with a thiol-oxidoreductase to facilitate oxidation of proteins *in vivo*. Based on the fusion of Trx and Erv domains for QSOX, it might be anticipated that a thioredoxin-like protein (*e.g.*, protein disulfide isomerase [PDI]) would be an efficient partner for Erv2. Overproduced Erv2 in yeast cells forms a mixed-disulfide bond with yeast PDI (Pdi1), suggestive of a transient association between the two proteins in cells (48). However, Erv2 shows limited activity toward Pdi1 *in vitro* (48, 57), and whether Pdi1 is a physiological substrate for Erv2 remains unclear (Fig. 6).

It is worth noting that neither Erv2 nor QSOX proteins show significant activity toward monothiols, including glutathione (29, 32, 35, 57, 59). *T. brucei* QSOX is also a poor oxidant for the trypanosome glutathione equivalent, the dithiol trypanothione (35). These data suggest that in cells Erv2 and QSOX are not likely to perturb the small molecule thiol redox pool. Instead, QSOX, and by inference Erv2, is likely an efficient oxidant of protein substrates *in vivo* and kinetically separated from interaction with the cellular glutathione pool.

Cellular Functions for Erv2

Current data favor a role for Erv2 as an oxidase within the ER lumen. Overproduced Erv2 localizes to the ER in yeast cells (48). Overproduced Erv2 can facilitate oxidation of ER proteins when oxidative protein folding in the ER is compromised, due to decreased activity of the major ER-resident oxidase in yeast, Ero1 (48). Deletion of the *ERV2* gene in an *ero1* mutant background increases cellular sensitivity to the reductant DTT (48), suggesting that the presence of *ERV2* normally augments the oxidizing power within the ER. However, a yeast strain deleted for the *ERV2* gene (*erv2Δ*) does not exhibit phenotypes associated with gross ER folding defects. For example, *erv2Δ* cells do not show sensitivity to reductant or folding defects for the disulfide-bonded protein carboxypeptidase Y (48, 55). Similarly, transcription of *ERV2* (or *ERVA* in *A. niger*) is not induced by global protein misfolding in the ER resultant from exposure of cells to the reductant DTT and is not a target of the unfolded protein response (UPR) (24, 54). Considering the ability of Erv2 to facilitate folding within the ER, yet lack of global folding defects upon loss of Erv2 activity, it has been suggested that Erv2 may contribute to disulfide formation in a subset of proteins or growth conditions (48, 55). Intriguingly, expression of the multiple-disulfide bond containing protein tissue plasminogen activator (tPA) in *A. niger* upregulates ErvA expression, suggesting that certain cellular folding requirements augment Erv2 expression, separate from the UPR (24). *Bona fide* physiological substrates for Erv2 remain to be identified, and studies focused on activity for endogenous (not overexpressed) Erv2 are warranted.

Considering the potential role for QSOX proteins outside of the ER (see next section), it can be argued that a role for Erv2 in cellular locations other than the ER should be examined. However, Erv2 is a poor direct oxidant of proteins *in vitro*, and no obvious candidates for partner oxidoreductases of Erv2 have been described outside the ER lumen. At present, it seems unlikely that Erv2 facilitates protein oxidation outside the ER unless Erv2 is found to be co-secreted with a partner enzyme.

Cellular Activities for QSOX

Distribution of QSOX1 in cell types with heavy secretory loads suggests that QSOX proteins likely facilitate protein oxidation within the secretory pathway (5, 43, 51). QSOX proteins have been detected within the ER (51, 56), Golgi (7, 38, 51, 56), and in the extracellular medium (2, 11, 39). The range of cellular locations reported for QSOX leaves open the question as to where in the cell QSOX operates. We will consider the data and potential for QSOX activity in distinct cellular locations individually. However, it should be noted that QSOX may function in a single or multiple cellular locations.

Like Erv2, overproduction of human QSOX1a (in yeast) can rescue the loss of viability and oxidative folding defects observed for yeast with impaired Ero1 function (7). In flies, a combined knockdown of *QSOX1* and *Ero1L* results in defects for Notch-related signaling (Notch being a heavily disulfide-bonded protein) (53). These data both show that QSOX can contribute to the oxidizing activity within the ER. However, in flies with Ero1L present, a loss of QSOX1 activity does not grossly compromise disulfide bond formation (53). In addition, overexpression of human QSOX1a in mammalian cells has little impact on disulfide bond formation of reduced tPA (7), arguing against essential or robust activity for QSOX1 within the ER. Like has been suggested for Erv2, QSOX may contribute toward ER oxidation of a subset of proteins or during specific cellular conditions. Alternatively, the ER-related activity observed for QSOX could be a product of its ability to function in the ER in transit to its steady-state locale. Whether the primary function for QSOX is within or outside the ER is difficult to assess. Proof requires a more direct indication of an ER folding defect in the absence of QSOX, such as a block in secretion for a reduced misfolded ER protein. An observed induction of the UPR upon QSOX knockdown would also suggest an ER function for QSOX. However, although induction of the UPR upon QSOX knockdown would suggest a role for QSOX in the ER, a lack of UPR induction upon knockdown would not rule out a selective role for QSOX in oxidative folding within the ER. Identification of QSOX substrates would greatly facilitate an understanding of QSOX cellular activity.

Arguably more intriguing than QSOX as a selective player in ER oxidation is the potential role for QSOX in post-ER oxidation of proteins, as suggested by the localization of QSOX to the Golgi and extracellular space. Although most proteins obtain their disulfides in the ER lumen during the folding process, there are proteins known to mature later in the secretory pathway into higher-order structures stabilized by disulfide bonds. For example, mucins assemble into disulfide-bonded multimers in the Golgi complex (44). Several observations also suggest a post-Golgi role for QSOX in the formation or remodeling of the extracellular matrix. QSOX1 levels are upregulated when human fibroblasts reach quiescence (10, 11), and increased expression during quiescence correlates with increased transcripts for extracellular proteins with structurally important disulfide bonds, like collagen and decorin (9). Similarly, transcript levels for QSOX in various tumor cell lines correlate most closely with levels for collagen type IV alpha 1 (46). QSOX proteins provide a complete protein oxidation pathway (Erv and Trx partners) that is physically tethered. The domain fusion in QSOX proteins may

allow for efficient disulfide formation outside of the cell, where it may be difficult to effectively concentrate individual Erv and Trx proteins.

QSOX, Growth Regulation, and Disease

Regulation of the cellular redox state through the oxidative activity of QSOX has potentially broad implications for normal cell growth and disease pathology. QSOX levels are repressed by estrogen in breast cancer and endometrial cells (31, 43), and downregulation of QSOX correlates with unregulated cell proliferation in breast and liver cancers (8, 41). Abnormal expression of QSOX has been shown also in pancreatic and prostate cancers (4, 49). With respect to cancer treatment, QSOX1 levels increase after treatment of cells with the antitumor drug gefitinib, an epidermal growth factor tyrosine kinase inhibitor (62). The correlation between an increase in QSOX1 levels and inhibition of proliferation after gefitinib treatment suggests QSOX1 could be a candidate for the modulation of gefitinib action (62). Most recently, it has been shown that QSOX1 facilitates pancreatic tumor cell invasion through Matrigel (33). It has been suggested that QSOX1 mediates pancreatic tumor cell invasion through direct or indirect posttranslational activation of matrix metalloproteinases (33).

QSOX may also play a positive role in relief from oxidative stress. Overexpression of QSOX attenuates a loss in cell viability and protects against apoptosis induced by oxidative stress in a human breast cancer cell line (42). Conversely, knockdown of QSOX by RNA interference in the malaria vector *Anophelis gambiae* results in a modest increase in mosquito mortality after ingestion of a blood meal, a source of oxidative stress for the mosquito (37). The effect of QSOX may be mediated by direct oxidation of cellular proteins that facilitate cellular protection against oxidative damage. Alternatively, it has been suggested that peroxide generated as a byproduct of QSOX activity could serve a signaling role, signaling the presence of oxidative stress and eliciting beneficial response systems (38).

Conclusions

Biochemical studies for Erv2 and QSOX proteins reveal unique structures and catalytic mechanisms for these related sulfhydryl oxidases. A crucial missing link in our understanding of Erv2 and QSOX proteins is how the sulfhydryl oxidase activity for these enzymes integrates with cellular physiology. More studies focused on Erv2 and QSOX in cells and also in the whole organism will provide insights into the roles for these proteins in growth and development. Notably, mouse ES lines with targeted disruption or gene trap alleles of QSOX1 and QSOX2 have been created (see www.informatics.jax.org/searchtool/Search.do?query=qsox*&submit=Quick+Search), yet phenotypic characterization of mice lacking either gene has not yet been described in the literature.

Before a true understanding of a cellular role for Erv2 and QSOX proteins can be obtained, it will be necessary to answer more definitively several fundamental questions including: Where are these enzymes physically localized within the cell? What proteins are substrates for Ev2/QSOX? When do these enzymes function during growth and development? How does loss of QSOX activity impact cell function? Tantalizing fragments of information exist related to each of these questions and suggest that interesting and important roles for these enzymes remain to be elucidated.

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Abbreviations Used

ALR = augmenter of liver regeneration
DTT = dithiothreitol
ER = endoplasmic reticulum
Erv = essential for respiratory and vegetative growth
FAD = flavin adenine dinucleotide
HRR = helix-rich region
IMS = inner membrane space
PDI = protein disulfide isomerase
QSOX = quiescin sulfhydryl oxidase
tPA = tissue plasminogen activator
Trx = thioredoxin
UPR = unfolded protein response

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