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FORUM REVIEW ARTICLE

Oxidative Protein Folding and the Quiescin–Sulfhydryl Oxidase Family of Flavoproteins

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Abstract

Flavin-linked sulfhydryl oxidases participate in the net generation of disulfide bonds during oxidative protein folding in the endoplasmic reticulum. Members of the Quiescin-sulfhydryl oxidase (QSOX) family catalyze the facile direct introduction of disulfide bonds into unfolded reduced proteins with the reduction of molecular oxygen to generate hydrogen peroxide. Current progress in dissecting the mechanism of QSOX enzymes is reviewed, with emphasis on the CxxC motifs in the thioredoxin and Erv/ALR domains and the involvement of the flavin prosthetic group. The tissue distribution and intra- and extracellular location of QSOX enzymes are discussed, and suggestions for the physiological role of these enzymes are presented. The review compares the substrate specificity and catalytic efficiency of the QSOX enzymes with members of the Ero1 family of flavin-dependent sulfhydryl oxidases: enzymes believed to play key roles in disulfide generation in yeast and higher eukaryotes. Finally, limitations of our current understanding of disulfide generation in metazoans are identified and questions posed for the future. *Antioxid. Redox Signal.* 13, 1217–1230.

Perspective

THE DISCOVERY of the Quiescin–sulfhydryl oxidase family is a story of color and serendipity. In 1979, Ostrowski *et al.* (71) were investigating major proteins from rat seminal vesicle secretions and noticed a yellow contaminant that they subsequently identified as a sulfhydryl oxidase (73). The Kistler laboratory showed that this 66 kDa monomeric FAD-containing flavoprotein was a vigorous generator of disulfide bonds capable of oxidizing a wide range of thiol compounds with the stoichiometry (72, 73):

$$2\ RSH + O_2 \rightarrow RS\text{-}SR + H_2O_2 \qquad \text{(Equation 1)}$$

The same activity had been previously suggested by Chang and Morton to play a role in the thiol/disulfide homeostasis in sperm (12). Some 25 years later, the flavoenzyme, now masquerading as a cysteine protease inhibitor (it was oxidizing the DTT included as a protective agent during protein purification), was found by Benayoun *et al.* to be homologous to a human growth factor designated Quiescin Q6 (6).

Our independent discovery of the Quiescin–sulfhydryl oxidase (QSOX) family started with the observation by White and coworkers that chicken egg white contains small amounts of FAD in addition to the abundant riboflavin used to support embryonic development (104). However the role of FAD was cryptic: FAD usually functions as a redox-active cofactor, but no flavin-linked enzyme had been described in avian eggs (104). We thus decided to purify the FAD-binding protein by following its yellow color (41). Once a homogeneous preparation was secured, attention turned to the identification of a possible enzymatic function for this protein. Hoober *et al.* found that reduction of anaerobic solutions of the egg white flavoprotein with sodium dithionite yielded a charge-transfer band, like that observed with a number of flavin-linked pyridine nucleotide-disulfide oxidoreductases (41). This association suggested that the egg white enzyme also had a flavin cofactor with a juxtaposed redox-active disulfide and this led to the identification of the protein as a flavin-dependent sulfhydryl oxidase (41). Sequencing of a number of peptides from the egg white oxidase showed convincing homology with several proteins annotated as human growth factors (40): in 1999 the best matches were bone-derived growth factor, cell growth inhibitory factor, and Quiescin Q6, a protein that was shown by Coppock *et al.* to be secreted from human fibroblasts as they approach confluence (15, 16, 40). Immunoprecipitates of Quiescin Q6 from conditioned media showed QSOX activity, and a polyclonal antibody raised against a Q6 peptide cross-reacted both with the avian oxidase, and a partially purified sample of sulfhydryl oxidase from bovine seminal vesicles (40).

Thus, the seminal vesicle (6) and egg white (40) oxidases represented founding members of the QSOX family of flavin-linked disulfide bond-forming catalysts. These multidomain enzymes are widely distributed throughout the eukaryotic

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evolutionary tree: from the smallest free-living eukaryotes to humans (17, 39, 92, 93). However, they are not found in fungi/yeast (93) and this has, to some extent, limited the attention they have received from the oxidative folding community at large. Nevertheless, the relative cellular abundance of the QSOX enzymes, their broad distribution, their vigorous catalytic reactivity, and their versatility as catalysts of *in vitro* oxidative folding systems, make them worth serious consideration as participants in the total cellular disulfide output (17, 39, 92, 93).

The discovery of the QSOX family is intertwined with the earliest reports of sulfhydryl oxidase activity stretching back more than a half-century. In 1958, Rony *et al.* coined the term "sulfhydryl oxidase" to describe an activity isolated from skin homogenates (83). The protein was later purified by Takamori *et al.* (91) and subsequently described as a copper-dependent oxidase (113). However, concerns with this suggestion were raised (9), and a skin QSOX was cloned (64) and found to be abundant in the outer epidermal layers (64, 92, 99). In 1967, a second sulfhydryl oxidase was partially purified by Kiermeier and Petz (51) from fresh bovine milk. The enzyme was of interest to the beverage and food industries because it counteracts the unpleasant taste associated with ultra-high temperature pasteurization (90). While the activity of early preparations of this protein were reported to be stimulated by added FAD (52), the milk enzyme was subsequently designated as an iron-dependent oxidase (47). However, a newly-developed purification procedure for the milk enzyme showed that lactoferrin, an iron-binding protein in milk, was a stubborn contaminant of the oxidase. Jaje *et al.* showed that essentially all of the sulfhydryl oxidase activity that could be recovered from skim milk comprised a typically yellow FAD-dependent QSOX (46).

While there have been other isolated reports of metalloenzyme sulfhydryl oxidases, none of them contain a detailed analysis of their metal centers and the role the metals play in catalysis. It will be interesting to revisit these earlier reports with the improved protein purification methods, bioanalytical procedures, and mass spectrometric sequencing methods that are now available. While the significance of these metal-dependent oxidases is seemingly clouded, there is no obvious reason why Nature should not have exploited the well-known propensity of certain transition metal ions to promote the autoxidation of thiols.

Sulfhydryl Oxidases and the Generation of Disulfide Bonds

Sulfhydryl oxidases are net generators of disulfide bonds (as in Equation 1). It is important to note that the protein disulfide isomerases (PDI) do not fit this description: for in their oxidoreductase mode they just exchange one disulfide for another. The common practice of describing PDI as an oxidase, or as a protein with oxidase activity, misrepresents the enzymological basis of oxidases defined decades ago: "oxidase will be used only for cases when O_2 acts as an acceptor . . ." (18). Describing PDI as an oxidase, inappropriately implying that PDI transfers electrons directly to molecular oxygen, also diverts attention from a central problem in the oxidative protein folding field—how to dispose of the pairs of electrons generated as each disulfide bond is introduced into a protein undergoing oxidative protein folding (92). This is an important issue, because a product of most of the flavin-linked disulfide-generating enzymes is hydrogen peroxide and its generation within cells with a heavy secretory load may lead to significant levels of redox stress (17, 37, 60, 98).

Disulfide Bond-Forming Reactions

Before we focus on the QSOX family of proteins, it is appropriate to reassert (36, 93), how little we know about the relative importance of any of the routes to the net generation of disulfide bonds in higher eukaryotes. The impression usually conveyed in the current literature is that disulfide bonds are generated in the endoplasmic reticulum (ER) of multicellular organisms by a single enzyme system, and that *Saccharomyces cerevisiae* represents a secure prototype to base our understanding of oxidative folding in metazoans. As a reminder of the diversity of potential disulfide-generating reactions in cells, this section presents a number of candidates.

The apparently facile nonenzymatic oxidation of protein thiols to disulfides in aerobic solution is catalyzed by copper or iron salts that are common, and largely unavoidable, trace contaminants of reagents and containers (Fig. 1A) (67). Without metal ion catalysis, the direct reaction between dithiols and oxygen is very slow because it is formally spin-forbidden: oxygen is a ground state triplet whereas thiols are ground state singlets. Reactions involving free radicals circumvent this spin restriction and can lead to oxygen-driven thiol consumption (108).

In contrast to oxygen, hydrogen peroxide can react with thiols in a spin-allowed reaction via the intermediacy of a sulfenic acid derivative (Fig. 1B). This thermodynamically favorable reaction has received renewed interest as a net contributor to disulfide bond generation (50). While this nonenzymatic reaction is comparatively slow (109), a number of thiol peroxidases and peroxiredoxins (50, 111) accelerate the reaction dramatically. The combination of sulfhydryl oxidase and thiol peroxidase would then generate two disulfide bonds for every oxygen molecule consumed (50).

Quinones (Fig. 1C) occupy a key position because of their role in the active site of the membrane-bound oxidoreductase DsbB driving oxidative protein folding in the periplasm of *Escherichia coli* (45, 49, 69). The reduced quinone is then reoxidized by the bacterial respiratory chain with the eventual reduction of oxygen to water or, anaerobically, by the conversion of fumarate to succinate. In bacterial species that lack DsbB, a related quinone has been suggested to be involved in the disulfide bond generation (20). Thus, bacterial homologs of vitamin K epoxide reductase (VKOR) can use the epoxide as the final electron acceptor in the generation of disulfide bonds (Fig. 1D). In higher organisms, reduced PDI is reoxidized by vitamin K epoxide generated during the γ-carboxylation of proteins (102). The oxidized form of vitamin C has been suggested to contribute to disulfide bond formation *in vivo* (5) via the reaction shown in Figure 1E. The reaction between dehydroascorbate and a range of thiol compounds, including reduced unfolded proteins and reduced PDI, has recently been critically reevaluated (61).

While dehydroascorbate can react directly with free thiols, the interaction with free flavins (Fig. 1F) is of no obvious physiological significance in oxidative protein folding. Indeed a significant nonspecific reaction between thiols and oxidized free flavin would present grave problems for an aerobe

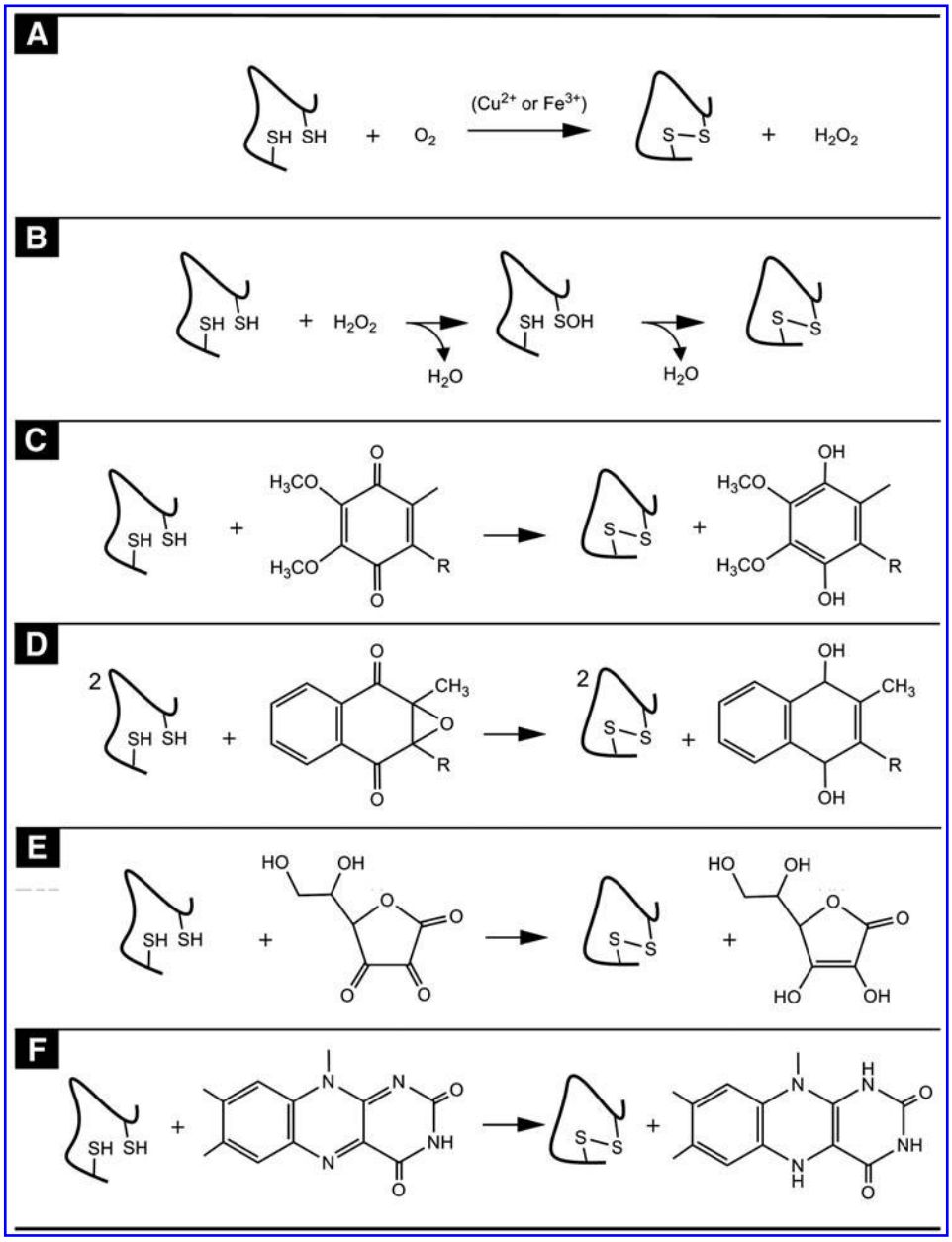

FIG. 1. Some reactions generating disulfide bonds. A number of transition metal ions, notably copper and iron, catalyze the oxidation of thiols to disulfides in aerobic solutions (**A**). Hydrogen peroxide is a slow, direct oxidant of free thiols (**B**). Quinones are used to oxidize thiols during catalysis by DsbB (**C**) and VKOR (**D**). Dehydroascorbate can accept reducing equivalents from protein thiols as shown in **E**. The flavoprotein sulfhydryl oxidases use the isoalloxazine ring to transfer reducing equivalents from reduced protein substrates to molecular oxygen (**F**).

because free reduced flavins are facilely reoxidized by oxygen leading to superoxide and hydrogen peroxide formation (63, 65). Fortunately the uncatalyzed reaction between thiols and free flavin is indeed very slow. Second, the cellular concentration of free flavins is maintained at a low level (reflected by the typically extremely tight binding of flavins to their apoenzymes). A low concentration of free flavin is also important to avoid the non-specific diaphorase activities of many flavoenzymes—their ability to transfer electrons to artificial exogenous acceptors (including free flavins) in competition with their physiological oxidants. While free flavins do not seem to be important participants in oxidative protein folding, when bound as a cofactor of flavin-dependent sulfhydryl oxidases, they provide the critical catalytic apparatus to connect thiols and molecular oxygen. In all sulfhydryl oxidases so far examined (including members of the Erv/ALR (23, 24, 33, 112), QSOX (6, 38, 39), and Ero families (31, 75, 87, 97)), this interaction is facilitated by a redox-active disulfide, typically a CxxC motif, contributed by the oxidase that lies immediately adjacent to the flavin ring (the proximal disulfide bond). The orientation of this critical disulfide bond with the isoalloxazine ring is illustrated for the yeast sulfhydryl oxidase Erv2p (33) in Figure 2. The interaction between these redox groups is described in the next section.

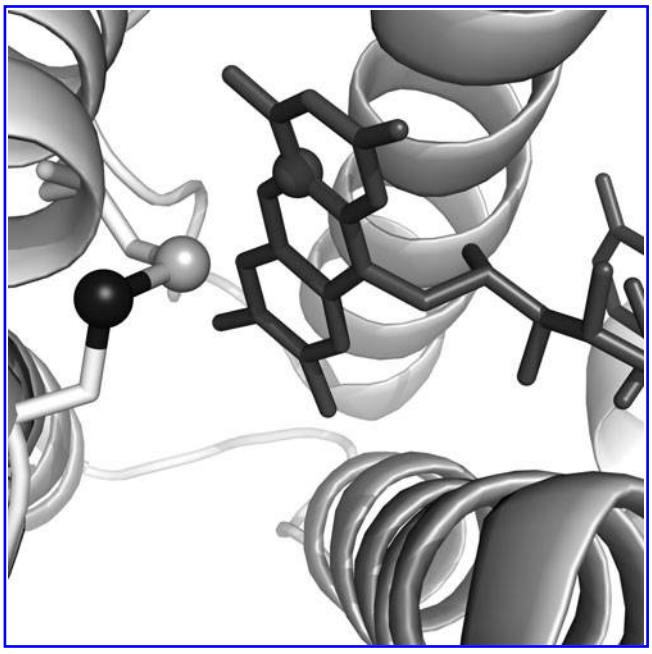

FIG. 2. The flavin ring of FAD and the proximal disulfide in Erv2p. The structure is taken from PDB 1JR8 (33). The C4a position of the isoalloxazine ring is indicated by a *dark sphere* and lies within van der Waals distance of the "charge-transfer" thiol of the proximal dithiol pair. The outer, or interchange, sulfur atom is indicated by a *dark sphere*.

Mechanism of a Model Flavin-Dependent Sulfhydryl Oxidase

Reduction of the model sulfhydryl oxidase (Fig. 3) is initiated by attack of a thiolate sulfur of a donor substrate on the outermost sulfur of the proximal disulfide (shown in **boldface**). It is not widely appreciated that disulfide exchange reactions (step B; and the corresponding resolution of the mixed disulfide in step D) have significant steric restraints. Thus, computational approaches suggest that these reactions proceed optimally when the attacking thiolate and the two sulfur atoms of the disulfide undergoing scission are co-linear (3, 25, 39). Indeed, this exchange between the model oxidase and a bulky thiol substrate seems only feasible (in the absence of significant conformational reorganizations) if the interchange thiol is solvent accessible and its enzyme partner lies buried below it in the protein matrix (depicted in Form 4; (39)).

The lower row of intermediates in Figure 3 represents transfer of the pair of reducing equivalents from the reduced proximal disulfide to the flavin cofactor. Studies with a range of flavoprotein disulfide oxidoreductases (107) show that thiolate to flavin charge-transfer complexes (*e.g.*, form 5) and C4a flavin adducts (*e.g.*, form 7) are intermediate in the eventual reduction of the flavin. Formation of this adduct requires concomitant protonation of the N5 position as in form 6 (19, 94). Overall, the acid and base catalysts implied by the series of transformations shown in Figure 3 are not yet known. Turnover is completed with the transfer of a pair of reducing equivalents from dihydroflavin to molecular oxygen to form hydrogen peroxide and to regenerate the oxidized enzyme (step I). Here, the flavin cofactor circumvents the spin restrictions that normally suppress the reactions of molecular oxygen with organic molecules by utilizing radical chemistry. However, the factors that modulate the reactions between flavin and oxygen are still not well understood (63, 65).

The minimal mechanism shown in Figure 3 is in many sulfhydryl oxidases supplemented with the inclusion of an additional redox-active disulfide that appears to be the primary site for donation of reducing equivalents from thiol substrates (24, 31, 39). This disulfide has been termed "distal" (as opposed to proximal) or as "shuttle," implying a functional role in a series of disulfide exchanges. In the QSOX family this shuttle disulfide is housed in a separate thioredoxin domain (see later) that must communicate with the

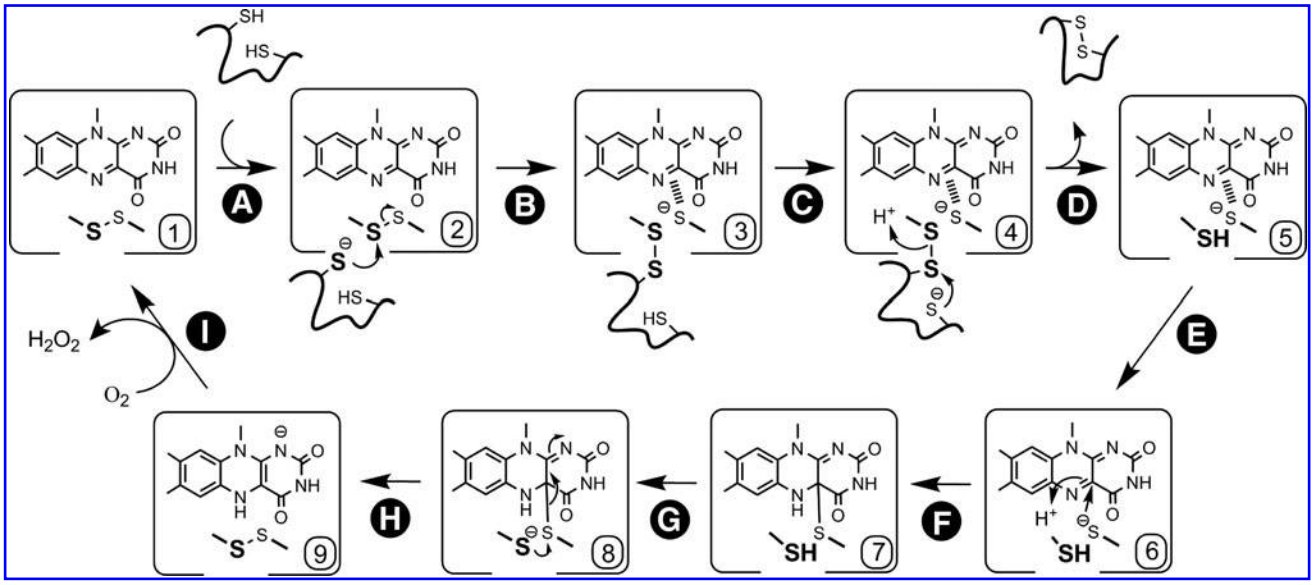

FIG. 3. Simplified reaction scheme for a generic sulfhydryl oxidase. A dithiol substrate is represented by a *wavy line*. The isoalloxazine ring of the bound flavin, and the adjacent redox-active disulfide, are included in the *boxes*. One of the cysteine residues forming this proximal disulfide interacts primarily with the flavin (the "charge-transfer" thiol). The second "interchange" cysteine (shown in **boldface**) forms mixed disulfides with external substrates, as in form 3.

Table 1. Catalytic Efficiency of Avian QSOX1

Thiol substrate	k_{cat} *(thiols oxidized/min)*	K_m *(mM thiols)*	k_{cat}/K_m $(M^{-1}\ s^{-1})$
GSH	2770	20.0	2.3×10^3
Cysteine	2550	10.9	3.9×10^3
DTT	2066	0.15	2.3×10^5
Reduced RNase	1220	0.12	1.8×10^5
Reduced lysozyme	1720	0.11	2.6×10^5
Reduced RfBP	2200	0.23	1.6×10^5
Reduced PDI	–	–	$<1\times10^2$

proximal disulfide in the Erv/ALR module depicted in Figure 2. As will be seen in the next sections, this distal disulfide exerts a profound influence on QSOX catalysis.

Substrate Specificity of QSOX Family Members

Avian (41–43), bovine (46), the human recombinant QSOX1 enzyme (38), and a *Trypanosomal* QSOX (Kodali and Thorpe; unpublished observations) show very similar specificity profiles and k_{cat}/K_m values for thiol substrates. Thus, where comparisons can be made, catalytic efficiencies between the enzymes for a given substrate differ by less than a factor of three. For illustration, Table 1 provides a sampling of thiol substrates of the avian enzyme (41, 42). The k_{cat} terms are largely similar, because they reflect an internal rate-limiting step subsequent to the initial delivery of reducing equivalents to the enzyme (43). Hence, catalytic efficiencies toward thiol substrates exhibited by those members of the QSOX family so far examined (38, 41–43, 46, 72) are dominated by the K_m term. Monothiols, like glutathione and cysteine (Table 1), are relatively poor substrates (with k_{cat}/K_m values some 50- to 100-fold lower than for the dithiol pairs of a typical unfolded reduced protein (41, 42)).

DTT is a useful model dithiol substrate for QSOX enzymes and shows catalytic parameters comparable to those of the unfolded reduced proteins (RNase, lysozyme, and riboflavin binding protein) shown in Table 1. All of these reduced proteins are significantly unstructured when freed of the constraints of their disulfide bonds. A measure of this flexibility comes from assessing the reactivity of these reduced proteins towards 5,5′-dithiobis(2-nitrobenzoate) (DTNB). The reactivity of the rather bulky and polar DTNB reagent towards protein thiols has frequently been used as a measure of their solvent accessibility. In our case, for example, the ensemble of thiols in reduced RNase (8 thiols) and RfBP (18 thiols) react with 2 m*M* DTNB, in the absence of denaturant, with half-times of about 15 msec and 60 msec, respectively (81). Reduced lysozyme is also considered to be unfolded (30). To summarize, all unfolded reduced proteins tested to date appear to be good substrates of QSOX family members. In contrast, two well-structured proteins containing multiple thiol residues appear to be either minimal or nonsubstrates of QSOX (42). However, this aspect needs to be reinvestigated because of the suggestion that QSOX enzymes may play roles in late-stage disulfide bond generation in the Golgi ((11), see later).

The last entry in Table 1 is important because of the abundance of reduced PDI in the ER, and the critical role PDI plays in oxidative protein folding (1, 36, 105). Reduction of the two redox-active CxxC motifs of oxidized PDI generates a 4-electron reduced protein which, unlike the other examples, is stable and well-structured so that it can fulfill its roles as an oxidoreductase in the ER. Reduced PDI is an extremely poor direct substrate of both the avian and human QSOX1 enzymes (82). As will be discussed later, this could allow the targeted oxidation of unfolded reduced protein substrates without collateral oxidation of the reduced PDI otherwise needed for disulfide isomerization (82).

Domain Structure of QSOX

We next address the domain structure of the QSOX family and our current understanding of the mechanism of these proficient catalysts of disulfide bond generation. Early pre-steady state kinetic studies of the avian QSOX assumed the presence of the two redox entities identified during reductive titrations of the enzyme: a flavin and a redox-active disulfide (41, 42). However, when QSOX protein sequences became available (6, 40), up to 4 potential redox-active groups could be identified: the flavin and three CxxC disulfide motifs (Fig. 4).

Panel A in Figure 4 depicts the domain organization of a typical metazoan QSOX: four recognizable domains are evident (39, 80, 92). Two thioredoxin domains are followed by a helix-rich region (termed HRR) of unknown function that is apparently only found in members of the QSOX family (38, 39). The fourth domain (Erv/ALR) represents the oxidative engine of QSOX catalysis since it connects the thiol/disulfide redox pool to molecular oxygen via the mediation of the bound FAD cofactor (24, 39, 92). Importantly, QSOXs from

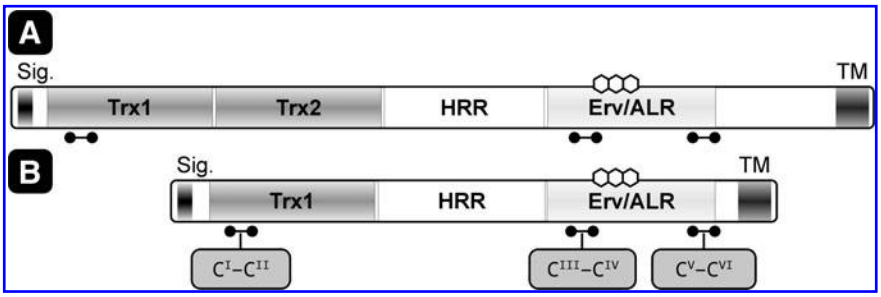

FIG. 4. Domain structure of one- and two-Trx QSOX enzymes. Metazoan QSOXs have two thioredoxin domains (labeled as Trx1 and Trx2 in **A**). Plants and protists lack all, or part, of the second thioredoxin domain (**B**). CxxC motifs are shown by the *solid circles* and identified in the text as C^{I}–C^{II}, C^{III}–C^{IV}, and C^{V}–C^{VI}. All QSOXs have a helix-rich region (HRR) fused to the flavin-binding domain (Erv/ALR).

certain protozoan parasites (*e.g., Trypanosoma*, *Schistosoma,* and *Plasmodia*), green algae, and higher plants preserve the same ordering of domains but lack all, or most, of the second thioredoxin domain ((17, 39, 92); Fig. 4B).

All QSOXs sequences start with a typical signal sequence almost immediately followed by the first thioredoxin domain. The closest non-QSOX relatives to the human QSOX Trx1 domain derive from protein disulfide isomerase (1, 34, 36, 95) and its analogs. The QSOX Trx1 domain has a typical redox-active CxxC motif predicted to be located at a strand helix intersection in the oxidase. This feature is catalytically critical: serving as the site of input of reducing equivalents (see later (38, 80)). While there is some variation in this thioredoxin CxxC motif, the most common variant, CGHC, is shared by many PDI sequences (39). The second thioredoxin domain, where present, lacks a CxxC motif. Its catalytic or structural functions are not yet known. The HRR domain, also of cryptic function, is predicted to be dominated by helices and appears to be the least conserved region in all of the 70 or so QSOX sequences available (39).

The Erv/ALR domain was recognized within the sequence of Quiescin Q6 (15) before any of these proteins were found to have sulfhydryl oxidase activity. Thus, Erv1p was originally identified as a growth factor found to be essential for respiration and vegetative growth in *S. cerevisiae* (55), while augmenter of liver regeneration (ALR) was discovered as a circulating growth factor in rats (26). Subsequently, Erv1p, ALR, and Erv2p were all found to be small dimeric FAD linked sulfhydryl oxidases (29, 54, 56, 85). The crystal structures of yeast Erv2p (33) and rat ALR (112) have provided key insight into the likely mechanism of QSOX enzymes. Figure 5 depicts our current understanding of the flow of reducing equivalents in QSOX using a subunit of Erv2p to depict the Erv/ALR domain of this multidomain protein (see later). While there are multiple recognized flavin binding folds in FAD-containing flavoproteins (21), the compact 4-helical bundle first reported by Fass and Kaiser and their coworkers (33) seems to have been reserved for flavin-linked sulfhydryl oxidases. Attention has been drawn to the similarity between the structure of DsbB with its bound quinone cofactor and the Erv/ALR fold (45, 86).

Following the Erv/ALR domain, a seemingly unstructured C-terminal region of unknown function is present in many QSOX family members. Where present, this region shows relatively little conservation in sequence. A transmembrane helix followed by a short cytoplasmic tail is present at the C-terminus of most QSOXs. Alternately spliced isoforms of QSOX lacking this transmembrane helix have been identified in certain metazoans.

Catalytic Mechanism of Metazoan QSOX

Recent studies with human recombinant QSOX1 (38) combined with earlier studies with the avian enzyme (41, 43, 80) have led to a model for catalysis represented in Figure 5. Three CxxC motifs are conserved in all QSOX sequences and are depicted in Figure 5A. The first (C^{I}–C^{II}) represents the redox-active disulfide of the Trx1 domain. In typical thioredoxin family members, the N-terminal cysteine (C^{I}, black circle in the Trx1 domain of Fig. 5B) is relatively solvent exposed and participates in mixed disulfide bond formation: first with the donor substrate and, upon disengagement, with the C^{III} of the proximal CxxC disulfide adjacent to the flavin cofactor in the Erv/ALR domain (Fig. 5B). Viewing the thioredoxin domain of QSOX as the substrate in the flavin reduction step, then steps B–D in Figure 3 represent the for-

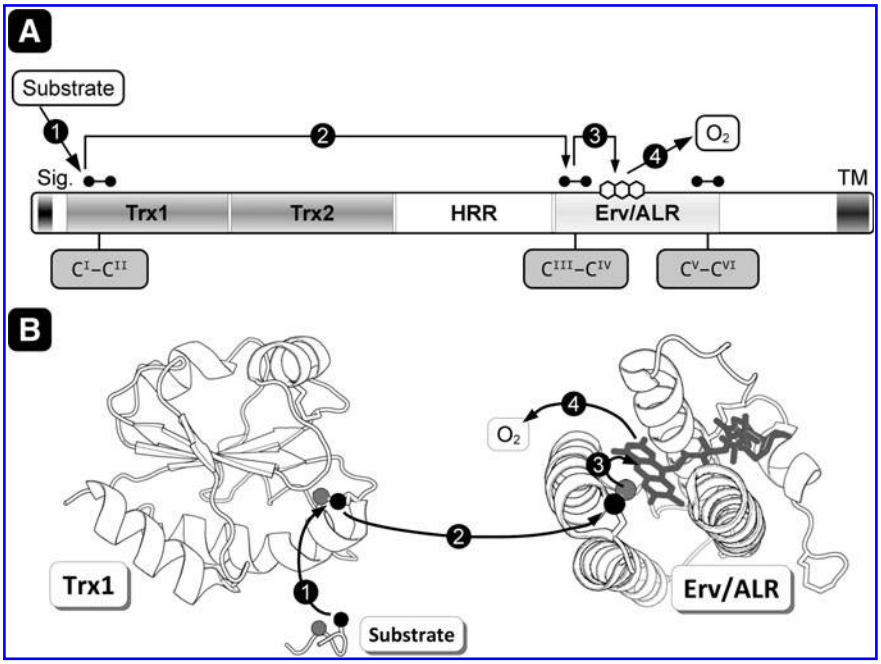

FIG. 5. Flow of reducing equivalents between the redox centers in QSOX. (**A**) shows the catalytic steps in the context of the domain structure of a metazoan QSOX. (**B**) depicts the interaction between thioredoxin and Erv/ALR domains using the crystal structures of the ***a*** domain of yeast PDI (95) and a subunit of Erv2p (33). The sulfur atoms participating in mixed disulfide bond formation (step 2) are shown as *dark spheres*.

mation and resolution of the mixed disulfide between the Trx1 and Erv/ALR domains. Upon reduction of the proximal disulfide (step D, Fig. 3), the C^{IV} thiol interacts with the oxidized flavin via charge-transfer and adduct formation with the eventual reduction of the flavin (steps F and G). The QSOX catalytic cycle is completed by 2-electron reduction of oxygen and the generation of hydrogen peroxide (step I (41, 72)).

The presence of a functional thioredoxin domain in QSOX is critical for the efficient oxidation of both DTT and reduced RNase. The removal of both thioredoxin domains in avian QSOX by partial proteolysis left the remaining fragment (encompassing HRR–Erv/ALR domains) unable to oxidize reduced RNase (80). The flavoprotein fragment retained low DTT oxidase activity (about 0.1% of the activity observed for the full length protein at its K_m for DTT (80)). Here, DTT can directly access the proximal disulfide of the Erv/ALR domain (Fig. 5). Low levels of activity were also observed when either C^{I} or C^{II} were mutated to serine in the Trx1 domain of human QSOX (38).

Mutations of the C^{III} and C^{IV} cysteines, comprising the proximal disulfide of the Erv/ALR domain, led to the expected loss of activity with both DTT and reduced RNase (38). As would be predicted from the crystal structures of members of the Erv/ALR families (24, 33, 112) the C^{IV} cysteine is involved in direct interaction with the flavin. Consistent with this expectation, mutation of C^{III} to an alanine or a serine residue isolates C^{IV} and allows it to form a long-wavelength charge-transfer band with the flavin (form 5, Fig. 3 (19)).

The C^{V}–C^{VI} disulfide was predicted to be catalytically essential (80) by analogy to the reaction mechanism suggested for yeast Erv2p (33). In this smaller dimeric oxidase, a CxC motif, found at a comparable position to the third CxxC disulfide in QSOX, functions as a redox shuttle, by conveying reducing equivalents from reduced PDI to the proximal disulfide (24, 33, 101). This internal disulfide exchange in Erv2p occurs across the subunit interface: from the CxC at the C-terminus of one subunit to the proximal disulfide of the other (24, 33). The observation that avian QSOX, like yeast Erv2p, was a homodimer was consistent with a comparable role for the C^{V}–C^{VI} disulfide in QSOX catalysis (80). Surprisingly, mutations of either, or both, of these cysteine residues in human QSOX1 had only marginal catalytic impact using either DTT or reduced RNase (38). The suggestion that intersubunit disulfide exchange is required for QSOX catalysis is also inconsistent with the observation that both the recombinant human and the rat seminal QSOX enzymes are monomeric, and the fact that the turnover number of the human enzyme is constant over a wide range of QSOX concentrations (38). Figure 5 incorporates these new findings and shows no catalytic role for C^{V}–C^{VI}. It is possible, but seemingly rather unlikely, that this disulfide functions in the oxidation of protein substrates other than reduced RNase. Alternatively this conserved terminal CxxC motif might play a regulatory role (38) or comprise part of a metal-binding site. It will be interesting to explore the consequences of this mutation *in vivo,* for example, when QSOX compensates for a loss of Ero1 activity.

Crystal structures of these multidomain QSOX enzymes should provide crucial insight and guidance for subsequent mechanistic inquiry. At this stage we know that a series of disulfide exchange reactions connect the oxidation of a pair of substrate thiols to the reduction of molecular oxygen via the mediation of the flavin prosthetic group. The fusion of one or two thioredoxin domains to the Erv/ALR domain appears to be of critical importance for the efficient oxidation of protein thiols by QSOX. It seems inevitable that this linkage requires some measure of flexibility between thioredoxin domain(s) and the Erv/ALR domains: the C^{I}-C^{II} of the Trx domain must first engage reduced protein substrates, disengage, and subsequently transfer reducing equivalents to the Erv/ALR domain. In all QSOXs so far examined, catalysis is limited by an internal redox equilibration: the rate limiting step occurs after the input of the first two reducing equivalents, but before the reoxidation of the enzyme by molecular oxygen. In terms of evolution, the fusion of Trx and Erv/ALR domains occurred in the eukaryotic lineage well before the fungi split from the branch leading to the metazoans. Hence, it appears that yeast/fungi lost QSOX in favor of a non-covalent complex between PDIs and Erv2p (24, 33, 85).

Our enzymological understanding of the QSOX family of proteins is far from complete. For example, it is currently unclear the extent to which unfolded reduced proteins bind to QSOX: what are the interaction energies and what are the lifetimes of these putative Michaelis complexes? Can QSOX function in a processive manner, catalyzing multiple turnovers within a reduced protein client before dissociation occurs? We also do not know the factors that influence whether any given protein is a substrate of QSOX. Another notable feature of QSOXs is the great multiplicity of intervening xx dipeptide sequences: in particular in the C^{III}–C^{IV} and C^{V}–C^{VI} CxxC motifs. There has been considerable interest in the influence of the intervening sequences in the CxxC motifs on the redox potential, and reactivity, of members of the thioredoxin superfamily (14, 78). It will be instructive to extend this inquiry to the CxxC motifs in the Trx and flavin-binding Erv/ALR domains of QSOX. A related question is how the redox potentials of the flavin and the C^{I}–C^{II}, C^{III}–C^{IV} disulfides are optimized for efficient catalysis in QSOX.

While these enzymological investigations are important to understand how QSOX has gained its catalytic proficiency over the single domain Erv/ALR family in the oxidation of protein thiols, it is critical to understand how these multidomain catalysts might participate in oxidative protein folding. The next section describes initial approaches to placing QSOX in a wider enzymological context.

Oxidative Protein Folding Catalyzed by QSOX and PDI

QSOX enzymes introduce disulfides into unfolded reduced proteins with turnover numbers averaging about 700 disulfides per minute and with K_m values of about 150 μM per protein thiol. This rapid generation of disulfides in RNase and in RfBP is not accompanied by the regain of native function of these client proteins (82). While this error-prone catalytic proficiency of QSOX has been suggested as a disqualification for a role in the ER (11), as far as we are aware no physiological oxidant is capable of generating the correct pairings *de novo* without the iteration that the PDI proteins provide. Because reduced PDI is not a significant direct substrate of QSOX (82), it functions in Figure 6A solely to isomerize mispairings introduced by the sulfhydryl oxidase. QSOX can therefore insert disulfide bonds into client proteins without collateral oxidation of PDI and without the consequent indirect oxidation of the glutathione redox pool ((82, 92) see later). Recently, nanomolar levels of QSOX and realistically high levels of

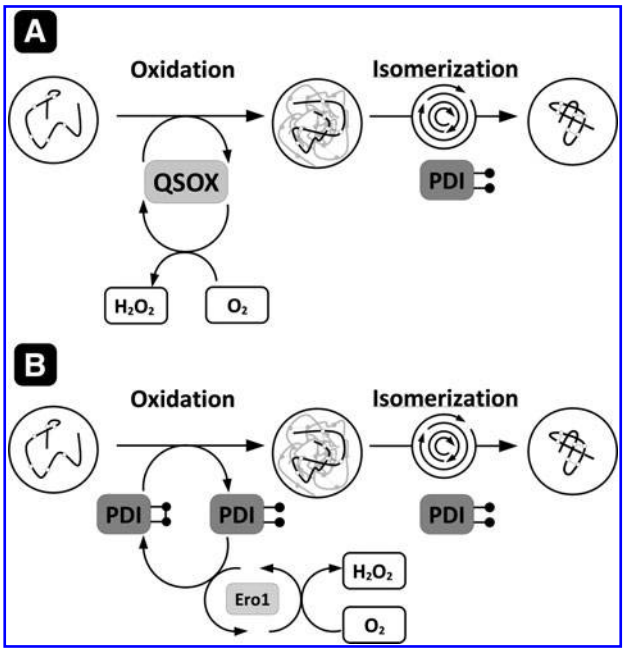

FIG. 6. Two models for oxidative folding catalyzed by sulfhydryl oxidases and PDI. QSOX oxidizes an unfolded protein directly and PDI isomerizes incorrectly paired disulfide bonds as they begin to accumulate (**A**). Oxidized PDI is the immediate oxidant for protein clients and is regenerated by Ero1-dependent reduction of molecular oxygen to form hydrogen peroxide (**B**). Reduced PDI is subsequently needed for the isomerization step.

reduced PDI (approaching the presumed concentration of this foldase in the ER) were found to be all that was necessary to efficiently refold a 9-disulfide-containing protein in aerobic buffer (82). The protein client for these experiments was RfBP (Fig. 7; regain of activity was assessed by fluorescence quenching as riboflavin rebinds to functional apoprotein). Since flavin binding to apo-RfBP occurs only after substantially all of the native pairings have been secured (76), this convenient continuous fluorescence assay provides useful insights into the factors that modulate the oxidative folding of proteins with complex disulfide connectivities.

The PDI concentrations in the vertebrate ER approach millimolar (48, 66, 105, 106) and under these conditions PDI cannot be regarded as just a catalyst—its concentration will be higher than any one of the client proteins it services. Thus, *in vitro* experiments that employ PDI at catalytic levels (at concentrations significantly lower than the client protein) are likely to underestimate contributions of the isomerase to modulating the global energetics of oxidative folding. For this reason, the refolding assay in Figure 7 utilized 1 μM reduced riboflavin binding protein and up to a 200-fold molar excess of reduced PDI (82). Using 30 nM QSOX, the maximal rates of regain of riboflavin binding showed half-saturation at 30 μM reduced PDI. When the concentration of QSOX was increased at fixed levels of PDI, refolding occurs progressively more rapidly until disulfide generation becomes too fast for PDI to keep up (82). Since fully oxidized mispaired ("scrambled") proteins appear to be poor substrates of PDI (28, 105), the isomerase would be expected to intervene before too many mispairings generated by QSOX accumulate to hinder overall folding. These *in vitro* experiments show that QSOX and reduced PDI can cooperate to efficiently generate a functional RfBP with only molecular oxygen as a co-substrate. No glutathione is required for this model oxidative folding system. Including 5 mM reduced GSH or a redox buffer of 5 mM GSH/1 mM GSSG slows the regain of flavin binding by approximately 2-fold (81, 82).

Comparison Between Two Models for Flavin-Dependent Oxidative Protein Folding

In Figure 6B, oxidized PDI serves as the immediate oxidant for client proteins and is recycled by the Ero1 proteins (2, 4, 10, 27, 66, 87, 97, 103) with the generation of hydrogen peroxide (32). Subsequently, reduced PDI (in its shuffling mode), or mixtures of reduced and oxidized PDI (if PDI is functioning by cycles of net reduction and reoxidation), serve to rearrange mispaired disulfides.

While this model has gained wide currency, it merits further enzymological scrutiny. The extant *in vitro* data suggest that Ero1 is not an efficient *in vitro* catalyst (in terms of k_{cat}/K_m) for the oxidation of reduced PDI. In a number of *in vitro* experiments, Ero1 is assayed under conditions that seem too reducing to be realistic for the ER (*e.g.*, assays conducted in the presence of 10 mM GSH without the oxidized component of the glutathione redox buffer system appropriate for the ER (4, 88)). Of course, the catalytic proficiency of an enzyme is frequently subservient to aspects of cellular function that are not usually the immediate focus of enzymologists. Nevertheless it would be interesting to compare steady state kinetic parameters for QSOX and Ero1 with their thiol substrates in the presence of comparable concentrations of a glutathione redox buffer approximating the redox poise of the ER lumen. It would also be a useful exercise to compare the

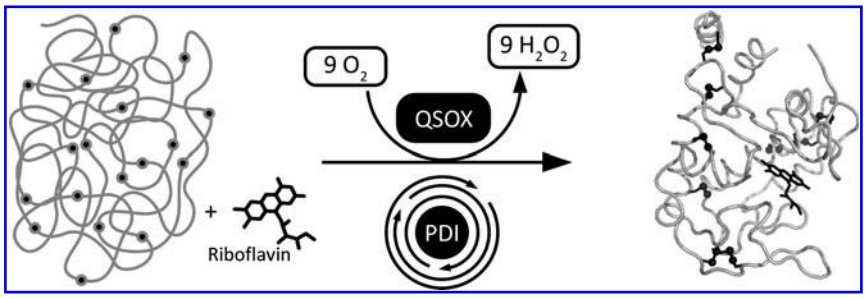

FIG. 7. Oxidative protein folding of riboflavin binding protein. Riboflavin binding to apo-RfBP is catalyzed by low nanomolar levels of QSOX and high micromolar concentrations of reduced PDI.

QSOX/PDI and PDI/Ero1 systems using the RfBP assay for oxidative folding (Fig. 7).

One interesting consequence of the model in Figure 6B is that it seemingly predicts a futile cycle that, if unchecked, would lead to the runaway consumption of cellular glutathione and the generation of hydrogen peroxide (82, 92). Figure 8 shows that GSH will compete with client proteins for the same oxidized PDI. Thus, the concentrations of GSH prevailing in the ER would lead to substantial reduction of oxidized PDI in less than a second (82) and to the net generation of hydrogen peroxide (as depicted in the lower part of Fig. 8). Perhaps this hydrogen peroxide might serve as the direct oxidant for oxidative protein folding in the ER via glutathione peroxidase or the peroxiredoxins (Fig. 1B) (50, 111). Alternatively, Ero1 might function in the context of a multienzyme assembly that could efficiently promote oxidative protein folding while being functionally segregated from the bulk redox pool of the ER lumen. Disruption of Ero1 expression in multicellular organisms leads to accumulation of misfolded proteins and upregulation of the unfolded protein response (35, 70, 84, 87). Clearly, Ero1 plays important roles in the net generation of disulfides and/or the regulation of ER redox poise (2, 4, 7, 87).

QSOXs from Protists to Humans

The consecutive fusion of thioredoxin, HRR, and Erv/ALR domains has yet to be found in any prokaryotic genome. QSOX sequences are also absent in the fungi (including *S. cerevisiae*, *Schizosaccharomyces pombe*, *Aspergillus nidulans*, *Neurospora crassa,* and *Encephalitozoon cuniculi* (17). However, a number of unicellular organisms contain a single QSOX gene including the small marine algae *Ostreococcus tauri* and several human protozoan parasites (*e.g., Plasmodium*, *Trypanosoma*, *Leishmania,* and *Cryptosporidia*) (17, 39). QSOX is not found in the early amitochondriate protist *Giardia intestinalis* or the slime-mold *Dictyostelium discoidum* (17, 93). Where present, protist and plant QSOXs contain a single complete Trx domain (Fig. 4B).

Two or more QSOX paralogs are found in higher plants (44) and in metazoans, with the animal enzymes all containing two consecutive Trx domains (see earlier). Multiple duplications of the QSOX gene have occurred during evolution (17, 93); *Drosophila* has 4 and *Caenorhabditis* has 3 paralogs. In vertebrates, the two QSOX forms appear to have been generated by a gene duplication that occurred about 450 million years ago (17, 93).

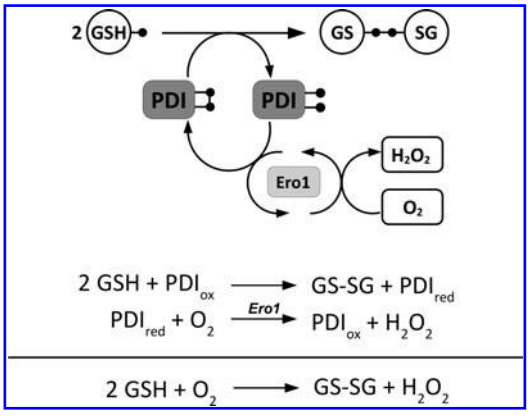

FIG. 8. PDI and Ero1 catalyze hydrogen peroxide formation at the expense of reduced glutathione. This system replaces reduced unfolded client proteins (Fig. 6B) with reduced glutathione in the ER lumen. These reactions could lead to unwanted loss of reducing equivalents as hydrogen peroxide (see text).

QSOX Proteins in Humans

All of the work described to this point refers to the generally most abundant form of QSOX in vertebrates (now given the official gene name QSOX1). Table 2 collects names and abbreviations that have been used in the scientific, database, and patent literature to denote human QSOXs. QSOX2 has yet to be characterized from an enzymological perspective, but it has been found by Wittke *et al.* to maintain the ability of neuroblastoma cells to undergo apoptosis (110).

In most human tissues, QSOX2 appears to be expressed at lower levels than QSOX1 (17); some 9-fold less represented when averaged over all of the tissues included in Unigene build #221 (Fig. 9). QSOX1 is conspicuously abundant in lung, but is found at relatively prominent levels in a wide range of tissues (including bone, connective tissue, kidney, and placenta). For comparison, Figure 9 shows the corresponding EST frequencies for Ero1L and Ero1LB. While EST frequencies cannot be taken as reliable indicators of protein expression, QSOX1 levels are, on average, higher than either of the Ero1 proteins.

All vertebrate QSOX gene sequences code for a full length protein with a single C-terminal transmembrane helical region (Fig. 4). The long form of QSOX1 (QSOX1a) would be expected to be confined to the luminal aspects of the secretory apparatus or bound to the outer face of the plasma membrane. An epitope-tagged version of human QSOX1a accumulated in the Golgi of CHO cells (11). A QSOX2 specific antibody showed a predominance of signal at the nuclear and plasma membrane surfaces of neuroblastoma cells (110).

Differential splicing of the QSOX1 message generates shorter QSOX forms (QSOX1b) that lack the transmembrane helix (58, 68, 93, 110). Certainly, some fraction of soluble QSOX is secreted and accumulates in extracellular fluids, as noted below. In addition, proteolysis within the variable C-terminal region of QSOX1a could release active oxidase from the membrane. The contribution of proteolysis to the release of soluble QSOX is currently uncertain.

Extracellular and Intracellular Locations of QSOX

QSOX is found in secreted fluids including milk (46), semen (6, 13, 40, 72), egg white (40, 41), tears (79), and blood serum (89, 114). QSOX is also released into growth media: for example,

Table 2. Names and Abbreviations Used for Human Quiescin-Sulfhydryl Oxidases

HUGO gene name	*Alternative names*
QSOX1	Quiescin Q6 (QSCN6) Quiescin Bone-derived growth factor Cell growth-inhibiting factor Placental-derived prostrate growth factor
QSOX2	QSCN6L1 SOXN

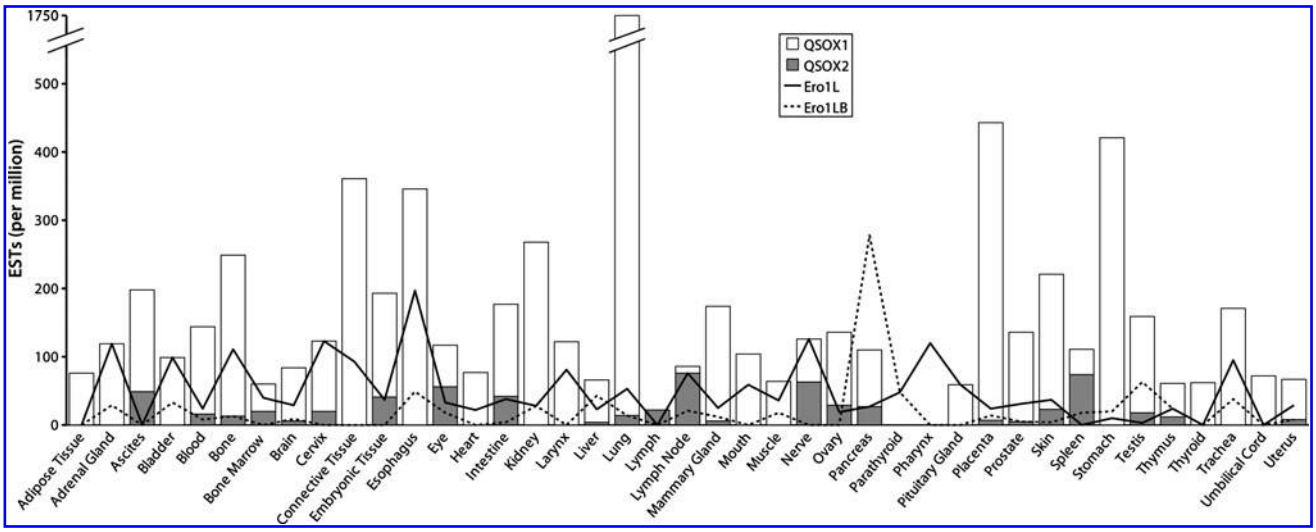

FIG. 9. EST frequencies for four sulfhydryl oxidases in human tissues. The data, from Unigene Build 221, are expressed as frequencies per million ESTs. The column heights represent the aggregate of QSOX1 and QSOX2 EST frequencies, with the *open* and *filled* components indicating the ratio of QSOX1 to QSOX2 frequencies, respectively. The *solid* and *dotted lines* represent the corresponding EST frequencies for Ero1L and Ero1LB. QSOX1 is particularly abundant in lung tissue (*broken vertical axis*). For comparison, a chondrosarcoma cell line shows a frequency of 6746 QSOX1 ESTs per million.

from human fibroblasts approaching confluence (15, 16), from CHO cells (6), and from breast (53), osteosarcoma (22), and prostate cancer cells (53, 62). The roles of these forms of QSOX are currently unknown. Perhaps oxidative folding continues outside the cell for some proteins (99), or secreted QSOX might participate in the assembly of structures that are too elaborate for completion intracellularly (11, 17). Secreted QSOX might also serve an antimicrobial function in lumenal fluids via the generation of hydrogen peroxide, perhaps sustained by the export of reduced glutathione (17, 72). An additional role for QSOX in hydrogen peroxide-mediated signaling in the nervous system has been suggested by Mairet–Coello *et al.* (57). More generally, QSOX, secreted from cells or confined to the outer face of the plasma membrane, might participate in extracellular redox cycles involving cellular adhesion, proliferation, and development (39, 59, 77, 92, 93).

Database correlations suggest a role for QSOX in extracellular matrix formation. In the NCI 60 tumor cell line database, the first and third proteins that most closely correlate to the expression levels of QSOX1 are collagen type IV alpha1 and lysyl oxidase (17). Consistent with a role in ECM generation, the level of QSOX1 mRNA in a chondrosarcoma, a tumor that is highly active in the generation of cartilage components, is almost 30-fold higher than those found in bone (legend to Fig. 9). Recent studies with *C. elegans* provides direct evidence for a role of QSOX in collagenous cuticular development (8). The *C. elegans* genome has three QSOX paralogs, with one of them (QSOX3) expressed in three alternately spliced isoforms. Disrupting the expression of one or more QSOX paralogs, either by means of RNAi or deletion mutations, results in mild to severe phenotypes related to the biosynthesis of collagenous proteins. A double knock out of QSOX1 and QSOX2 is lethal in *C. elegans* (8).

Intracellularly, QSOXs have been reported in the ER (93, 100), in the Golgi (11, 57, 93, 100), secretory granules (57, 100), and QSOX2 at the nuclear and plasma membranes (110). A key issue that remains to be established is the relative abundance, and catalytic contribution, of soluble and membrane-bound forms of QSOX.

Flavoproteins in Oxidative Protein Folding

Recent studies by Bellen and coworkers (96) highlight how much more we need to know about the enzymology of oxidative protein folding in metazoans. Lack of the only Ero1L in *Drosophila* does not lead to adult cellular lethality and produces a surprisingly mild phenotype in the Notch signaling pathway (96). The Notch receptor has approximately 120 structural disulfides in its extracellular domain, and is likely a sensitive indicator of impaired oxidative protein folding. Knockdown of one of the four paralogs of *Drosophila* QSOX (CG4670; the other three are located almost exclusively in the male accessory glands) had no noticeable effect on the fly, but a double knockdown exacerbates the Ero1L phenotype. Ero1L knockdown had no discernable impact on the other developmental pathways selected for study; a finding consistent with degeneracy of pathways for disulfide bond generation in *Drosophila*. Finally, experiments with cultured insect cells suggested, somewhat surprisingly, that QSOX1 and Ero1L insert disulfides into different domains of the Notch receptor: QSOX1 for the 36 EGF modules and Ero1L for the 3 LNR domains (96).

These interesting studies suggest that caution should be exercised before assuming the primacy of one particular mode of disulfide bond generation over other pathways. In terms of the Ero1 family, a series of elegant studies on the cell biology of these flavoproteins have appeared over the last few years, but we know much less about their enzymology and substrate specificity. The situation is just the opposite for QSOX. These multidomain enzymes have been the subject of a number of enzymological investigations. QSOX has been shown to cooperate with PDI to efficiently fold proteins with

complex disulfide connectivity *in vitro*. However, evidence for the physiological roles of any QSOX enzyme remains sparse.

This review has covered the discovery of the first QSOX enzymes, some 50 years ago, and has summarized more recent insights into their structure and mechanism. It has described *in vitro* experiments that suggest that the QSOX enzymes are orders of magnitude more efficient catalysts of disulfide bond generation than the Ero1 proteins. This catalytic proficiency is apparently paired with a several-fold greater average abundance of human QSOX1 over Ero1L and Ero1LB proteins (Fig. 9). A currently undetermined fraction of QSOX proteins are synthesized as longer splice-variants that can be anchored to the membrane via a C-terminal transmembrane helix. While little is known concerning their physiological roles, Bulleid and coworkers have shown that the long form of human QSOX1 accumulates in the Golgi when expressed in mammalian cells, and that overexpression of this form in yeast cells restores disulfide bond formation in carboxypeptidase Y (11). The shorter, soluble QSOX forms lack an obvious ER retention signal (17). Providing that these truncated versions are not retained by an association with ER-resident proteins (as has been described for human Ero1L and Ero1LB (74)), they are likely to be secreted.

The recognition that some fraction of QSOX is secreted seems to have deflected attention from a possible role of the oxidase in intracellular disulfide bond generation. One issue for inquiry is the extent to which these truncated forms of QSOX are active in disulfide bond generation as they transit the ER. A related point is whether the immunohistochemical methods used to establish cellular location are of sufficient sensitivity to quantitate low, steady-state, levels of soluble QSOX forms within the ER lumen. Modest QSOX concentrations may be all that is required *in vivo* because efficient catalysis of oxidative protein folding requires only low nanomolar levels *in vitro*. Currently, there seems to be no obvious reason why soluble QSOX proteins might not participate in disulfide generation in the ER prior to serving additional roles outside the cell. A prediction was raised during editorial review that addresses the site of QSOX action. If QSOX is active in the ER, any QSOX knockout phenotype would be expected to be associated with upregulation of the unfolded protein response, provided that the misfolded proteins accumulate in the ER. However, if QSOX activity is associated with a post-ER compartment, an enhanced unfolded protein response would not be expected.

While this review has centered on flavin-dependent sulfhydryl oxidases, we close by reiterating that these enzymes may only generate a subset of the disulfides that stabilize the array of proteins secreted by higher organisms. Hopefully, the next few years will see a more searching, and inclusive, evaluation of all models of disulfide bond generation: pairing cell biological investigations with a critical scrutiny of chemical feasibility and kinetic competence.

Acknowledgments

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Abbreviations Used

ALR = augmenter of liver regeneration
DTNB = 5,5′-dithiobis(2-nitrobenzoate)
ECM = extracellular matrix
ER = endoplasmic reticulum
Ero1L and Ero1LB = alpha and beta paralogs of mammalian endoplasmic reticulum oxidoreductin, respectively
ERV1p and ERV2p = proteins originally termed essential for respiration and vegetative growth
PDI = protein disulfide isomerase
QSOX = Quiescin-sulfhydryl oxidase
RfBP = riboflavin-binding protein
Trx = thioredoxin

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