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Forum Review

Multidomain Flavin-Dependent Sulfhydryl Oxidases

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ABSTRACT

Eukaryotic flavin-dependent sulfhydryl oxidases catalyze oxidative protein folding with the generation of disulfides and the reduction of oxygen to hydrogen peroxide. This review deals principally with the *Q*uiescin-*s*ulfhydryl *ox*idases (QSOX) that are found in multiple forms in multicellular organisms and singly in a number of protozoan parasites. QSOX is an ancient fusion of thioredoxin domains and an FAD-binding module, ERV1/ALR. Interdomain disulfide exchanges transmit reducing equivalents from substrates to the flavin cofactor and thence to molecular oxygen. The *in vitro* substrate specificity of avian QSOX1 and the likely substrates of QSOXs *in vivo* are discussed. The location of QSOX immunoreactivity and mRNA expression levels in human cells and tissues is reviewed. Generally, there is a marked association of QSOX1 expression with cell types that have a high secretory load of disulfide-containing peptides and proteins. The abundance of sulfhydryl oxidases in the islets of Langerhans suggests that oxidative protein folding may directly contribute to the oxidative stress believed to be a factor in the progression to type II diabetes. Finally, the structure and mechanism of QSOX proteins is compared to their smaller stand-alone cousins: yeast ERV1p and ERV2p, the mammalian augmenter of liver regeneration (ALR), and the viral ALR homologs. *Antioxid. Redox Signal.* 8, 300–311.

INTRODUCTION

THIS REVIEW CONCERNS a class of sulfhydryl oxidases that catalyze the net generation of disulfide bonds at the expense of molecular oxygen:

$$2\ \text{P-SH} + \text{O}_2 \rightarrow \text{P-S-S-P} + \text{H}_2\text{O}_2$$

These enzymes were first suggested as participants in vertebrate oxidative protein folding some 30 years ago (43, 46). At that time the fate of the 2-reducing equivalents that accompany the generation of each disulfide bond did not attract general interest. The vertebrate system was then eventually overshadowed by the elegant dissection of oxidative protein folding in *Escherichia coli* and yeast (44, 66, 78, 86). However, the discovery of a new flavin-dependent sulfhydryl oxidase (QSOX) from egg white (37), and the realization that both avian and seminal vesicle enzymes (6, 36) were members of a widely-distributed protein family, has led to renewed interest in these redox catalysts. QSOX family members include a quiescence-induced gene product (16), bone-derived growth factor (24), a cell growth inhibiting factor (73), an E2-regulated gene product in guinea pig (65), and an activity that stimulates apoptosis in a neuroblastoma cell line (98). These varied activities suggest that QSOX enzymes may have vital and pleiotropic effects on both cellular and organismal physiology.

Several classes of FAD-dependent enzymes with sulfhydryl oxidase activity have now been described, including the ERV1/ALR family, initially uncovered by Lisowsky (26, 57), the ERO class discovered independently by Kaiser (21, 22) and Weissman (70) and their colleagues, and the larger sulfhydryl oxidases which form the focus of this review. Although we will deal mainly with the QSOX enzymes, their smaller ERV1/ALR relatives will be briefly mentioned, together with the rather poorly understood metal-dependent sulfhydryl oxidases. The important ERO system is the subject of separate contributions in this series and will only be addressed peripherally. We will not deal with a glutathione oxidase found in *Aspergillus* and *Penicillium* sp. that is weakly related to a bacterial thioredoxin reductase (17, 49, 83). The gene context of this

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flavoenzyme suggests that it may be involved in the biosynthesis of gliotoxin and other epipolythiodioxopiperazine secondary metabolites (8, 25). Finally, a discussion of nonprotein oxidants that may also contribute to the net cellular output of disulfides (83) is beyond the scope of this review.

DISCOVERY, DOMAIN STRUCTURE, AND PHYLOGENY OF THE QSOX FAMILY OF ENZYMES

Two serendipitous observations (the discovery of low levels of FAD in egg white (37, 95) and the investigation of an apparent papain-inhibitory factor in seminal vesicle fluid (6)) led to the identification of a family of sulfhydryl oxidases formed from the fusion of thioredoxin and ERV1/ALR domains (6, 36). This arrangement of domains was initially recognized in Quiescin Q6 (QSCN6), whose expression was sharply upregulated when human fibroblasts approach quiescence (16). Quiescin was subsequently found to have sulfhydryl oxidase activity (36).

Domain structure

The typical arrangement of domains and structural features found in QSOX enzymes from *Caenorhabditis elegans* to vertebrate QSOXs is depicted in Figure 1. The numbering is for the avian QSOX1—currently the best understood member of this sulfhydryl oxidase family. QSOX sequences start with a signal sequence, consistent with location within, or transit through, the endoplasmic reticulum (ER; see later). Next, is a thioredoxin domain containing a CxxC redox-active motif (frequently the PDI-like WCGHC sequence). Figure 1 shows a second, more weakly-scoring, thioredoxin domain (TRX2) lacking the CxxC feature. The intervening "spacer" region shown in Figure 1 shows some weak non-QSOX Blast hits. Of these, the most intriguing represents the C-terminal 50-residue sequence just prior to the ERV1/ALR domain. This segment is 35% identical to an extracellular portion of a high-affinity peptide transporter from *C. elegans*. Possibly this stretch reflects a binding determinant for peptides and unfolded protein substrates of QSOX (see later).

The ERV1/ALR domain (Fig.1) drives catalysis in QSOX. ERV1p was first reported by Lisowsky and colleagues as a protein essential for respiration and viability in yeast (56). Subsequently ERV1p (53), ERV2p (26, 77), and augmenter of liver regeneration ALR (57) were shown to be diminutive flavoproteins with weak sulfhydryl oxidase activity. The important crystal structure of ERV2p by Fass and coworkers (29) revealed a new helix-rich FAD binding motif (Fig. 2A). ALR was shown by Wu et al. to have a similar fold (99) (Fig. 2B).

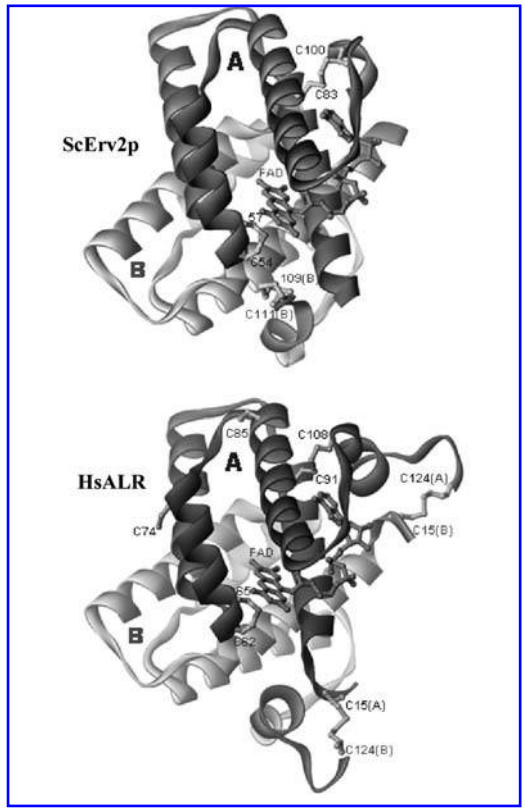
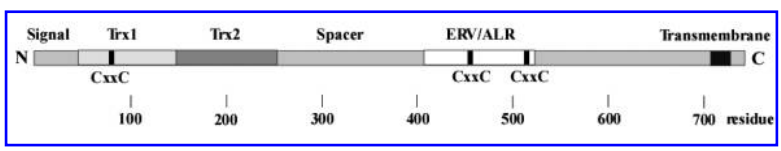


FIG. 2. Structures of the ERV2p and ALR dimers. The *top panel* shows the crystal structure of ERV2p from *Saccharomyces cerevisiae* (29). The redox-active distal disulfide (CxC; C109/111) of subunit B, the proximal disulfide (CxxC; C54/57), the structural disulfide (C83/100) and the FAD moieties of subunit A are depicted in a *ball and stick* representation. The sulfur atom of C57 forms a C-4a adduct with the flavin (see text). The *bottom panel* is a homology model of the human short form of ALR built from the coordinates of the rat ALR structure (99) (see text). The redox-active proximal disulfide is C62/65. Apparent structural disulfides are the intrasubunit C91/108 and the intersubunit C15/C124. Only one set of redox centers within the homodimer is shown for clarity.

Following the ERV1/ALR domain is a second spacer region within a highly variable C-terminal stretch of about 200 amino acid residues which terminates with a single transmembrane span (Fig. 1). None of the QSOX1 or QSOX2 enzymes examined to date has a KDEL retention signal. However, avian QSOX1 has a C-terminal F**R**L**RT**R**A**R**K**GR sequence consistent with three copies of an RXR arginine-based ER retention/ retrieval signal (82, 101). A shorter form of QSOX (of about 64 kDa) is encoded by an alternatively spliced message (60, 65, 83, 98). Alternatively, truncated

FIG. 1. Schematic depiction of the domain structure of avian QSOX. The domains are drawn approximately to scale. The *heavy vertical lines* represent the three redox-active CxxC motifs. The *solid box* at the right is a single transmembrane span with the C-terminus facing the cytoplasm.



forms of QSOX could be generated by proteolytic removal of the transmembrane span.

Phylogenetics

An examination of the current GenBank database with nearly complete genomes shows that all metazoans have at least one QSOX; that higher plants also have QSOX enzymes; and that several protozoan parasites of importance in human disease have a single QSOX-like gene including *Plasmodium falciparum* (malaria), *Trypanosoma brucei* (sleeping sickness), *Leishmania major* (leishmaniasis), and *Cryptosporidium parvum* (cryptosporidiosis). Importantly, the fungi including *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Aspergillus nidulans*, *Neurospora crassa,* and *Encephalitozoon cuniculi* (a microsporidian) do not have a QSOX, but rather have one or more copies of ERV1/ALR (see below). Metazoans appear to have duplicated the QSOX gene several times, since all of the *C. elegans* and *Drosophila melanogaster* QSOX paralogs are more related to those of the same species than to others. For the vertebrate clade, a duplication event apparently predated the division of bony fish from our mammalian and avian ancestors resulting in two forms of QSOX, QSOX1 (a.k.a. QSCN6) and QSOX2 (a.k.a. QSCN6L1, SOXN). A detailed comparison of the sequences of human QSOX1 and QSOX2 is informative (98).

The general arrangement of domains in human QSOX1 and QSOX2 is compared in Figure 3 with one (out of four) *Drosophila*, one (out of three) *C. elegans*, and one (of two) *Arabidopsis* QSOX sequences. The single *Trypanosoma brucei* sequence is shown at the bottom of the figure. All metazoan QSOXs examined show very similar domain organization including two fused thioredoxin domains. In contrast, plant (40, 83) and protist QSOXs (83) are significantly shorter, and retain only one thioredoxin domain (Fig. 3). The evolutionary origins of these differences, and their mechanistic consequences, await investigation. All QSOXs retain a highly helical spacer region between thioredoxin and ERV1/ALR domains.

ROLES OF DOMAINS IN CATALYSIS OF DISULFIDE BOND INSERTION

Partial proteolysis with chymotrypsin cuts the avian QSOX into two major pieces (71): the N-terminal portion contains both TRX domains and has no sulfhydryl oxidase activity. The larger C-terminal piece contains the ERV1/ALR domain, retains bound FAD, and shows very weak sulfhydryl oxidase activity with the artificial reagent DTT (some 1000-fold lower in k_{cat}/K_m—to a value comparable to that reported for ERV2p). This fragment shows undetectable sulfhydryl oxidase activity towards reduced RNase (71). Importantly, the chemically reduced TRX-containing domain is a substrate of the ERV1/ALR fragment. These data underscore the catalytic advantage that QSOX exploits with the fusion of a PDI-like thioredoxin domain to the ERV1/ALR oxidase module.

In QSOX a series of disulfide exchanges starts with the initial oxidation of a dithiol substrate by a relatively oxidizing C80-C83 disulfide housed in the first thioredoxin domain (Fig. 4, arrow 1). The initial substrate-QSOX mixed disulfide is likely formed between the solvent-exposed N-terminal cysteine of the TRX1 disulfide (here, C80) and a substrate thiol. Following resolution of this mixed disulfide the C80 thiolate would then be free to initiate a series of internal disulfide exchanges starting with arrow 2 in Figure 4. The placement of steps 2–4 relies on key insights gained from the crystal structure of ERV2p (29). Reduced yeast PDI (PDI1p) is believed to interact with the distal CxC motif at the flexible C-terminus of each subunit of dimeric ERV2p (highlighted in green in Fig. 2A)(29). Mixed disulfide bond formation between ERV2p and PDI1p has been observed in yeast constructs that overexpress both proteins (77). A comparably placed CxxC disulfide (C519–522 in avian QSOX1) is conserved in all QSOX sequences available to date and is the likely recipient of reducing equivalents from the fused N-terminal TRX domain ((83); step 2, Fig. 4). Reduction of the proximal disulfide in ERV2p occurs across the subunit interface ((29); Fig. 2A and Fig. 4). Given the convincing homologies with ERV2p, a corresponding intersubunit disulfide exchange (step 3, Fig. 4) would be expected in QSOX (29, 83). Thus the active form of QSOX would be expected to be dimeric. While the avian enzyme and an ERV1/ALR-containing proteolytic fragment thereof are dimers (37, 71), the rat seminal vesicle enzyme (RnQSOX1) is reported to run as a monomer on gel-filtration (67). Presumably the latter enzyme requires dimerization for maximal activity.

Transfer of two reducing equivalents to the disulfide C459/462 generates a cysteine thiolate C462 which has a low pK and participates in a strong charge-transfer interaction with the electron-deficient oxidized flavin ((39, 83); Fig. 5B). These features are common to a number of flavoenzymes in

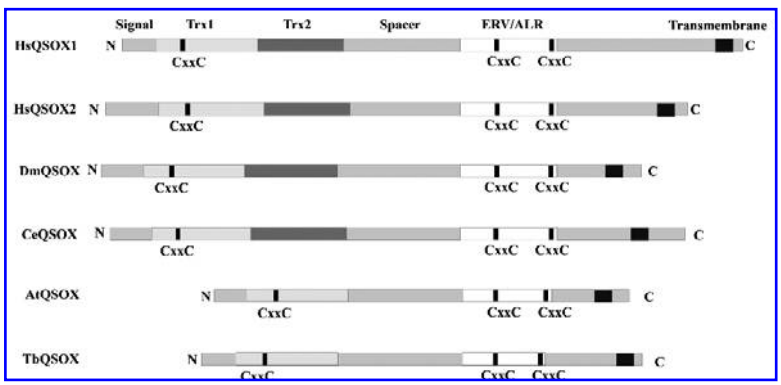


FIG. 3. Domain structure of selected QSOX enzymes. The two human QSOXs are shown, together with one (AE003819.3) of four *Drosophila melanogaster* and one (NP_508652.1) of three *Caenorhabditis elegans* proteins. As examples of nonmetazoans QSOXs, one (AY035175.1) of the two *Arabidopsis thaliana* proteins and the single *Trypanosoma brucei* QSOX are depicted.

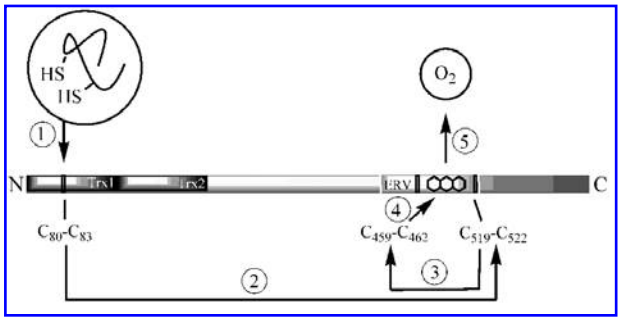


FIG. 4. Schematic representation of the proposed flow of reducing equivalents in QSOX catalysis. Starting with fully oxidized QSOX, a series of disulfide exchanges commencing with the oxidation of a dithiol substrate (*arrow 1*) culminates in reduction of the C459-C462 disulfide proximal to the flavin (*arrow 3*). This latter step occurs across the dimer interface (see text). Generation of dihydroflavin (step 4) is followed by 2-electron reduction of oxygen (step 5) with the liberation of hydrogen peroxide.

which the flavin prosthetic group is in redox communication with one or more disulfides (97). This nucleophilic thiolate then attacks the flavin to generate a C-4a adduct which ultimately yields reduced flavin and the reappearance of the proximal disulfide bond (83, 84, 97). Although the charge-transfer species can be simply represented as retaining two of three disulfides in QSOX (Fig. 5B), a contribution from an alternate pattern of disulfide connectivity appears probable (Fig. 5C). This would allow for thermodynamic coupling between the flavin and other disulfide centers. Evidence consistent with such an alternative comes from experiments with avian QSOX (71) and from an interesting study by Lisowsky and colleagues in which selected cysteine to serine mutations generated intense thiolate to flavin charge transfer features in yeast ERV1p (33).

Catalysis is completed via interaction of reduced flavin with oxygen (Fig. 4, arrow 5): first via 1-electron transfer and then via radical collapse to generate a C-4a hydroperoxyflavin intermediate (62). Such reactions would be expected to require an adequately polar local flavin environment to stabilize the incipient superoxide anion (91) and space to accommodate a C-4a adduct during peroxide generation (62). Fass and co-workers have identified such a channel in ERV2p through which oxygen can approach the isoalloxazine ring (28, 29).

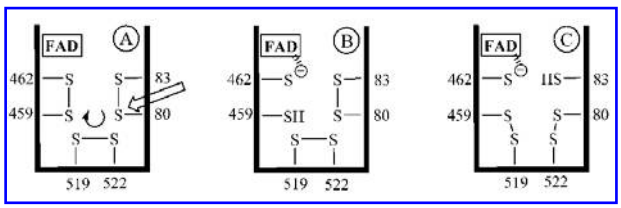


FIG. 5. A schematic representation of the four redox centers in QSOX catalysis. (**A**) Mixed disulfide bond formation with a substrate likely involves C80 (*arrow*) and a series of disulfide exchanges (*clockwise arrow*) yields a 2-electron reduced species showing a charge transfer absorption between the C462 thiolate and the oxidized FAD prosthetic group. (**B**) and (**C**) represent two isomeric arrangements of this 2-electron reduced enzyme.

SUBSTRATE SPECIFICITY

Currently avian QSOX1 is the only enzyme for which detailed enzymological studies have been done (35, 38, 39). Thiol substrates show a limiting k_{cat} value of about 1000/min reflecting an internal rate-limiting redox step (39, 71). Catalytic efficiency is then dominated by the K_m term. Preferred substrates of avian QSOX1 are not small monothiols (e.g., glutathione, K_m 20 m*M*), but rather an apparently unlimited array of reduced proteins and peptides (with typical K_m values per thiol of about 150–200 μ*M* (38)). There is little dependence on the molecular weight or the pI for these reduced protein substrates.

Protein substrates for QSOX are typically prepared by reduction of the multiple disulfide bridges of a secreted protein with consequent destabilization of its native structure. Pancreatic RNase is widely used because it shows a minimal tendency to aggregate upon reduction. Here the K_m value normalized per protein thiol is about 90 μ*M* for avian QSOX1 (38, 39). This facile oxidation does not generate the correct pairings (39), but rapid regeneration of RNase activity was achieved with the inclusion of PDI and low levels of reduced glutathione in the buffer (38). While this important result needs to be extended to a wider range of substrate proteins and incubation conditions, it is clear that avian QSOX1 is a facile and promiscuous disulfide-generating catalyst.

The substrate specificity of other QSOX enzymes remains to be investigated in depth. The seminal vesicle enzyme is active toward a range of small molecular weight mono- and dithiols with K_m values from 0.7 to 32 m*M* (4 m*M* for GSH) and k_{cat} values from 40,000 to 60,000/min (67). The latter rates are some 50-fold faster than the egg white protein (37, 38). Although the seminal QSOX enzyme can catalyze refolding of reduced RNase, the kinetic parameters for this process have not been established (67).

LOCATION AND ABUNDANCE OF MAMMALIAN QSOX ENZYMES

Immunohistochemistry

Two major mRNAs encode distinct predicted human QSOX1 proteins (604 aa and 747 aa) with an alternative splice site in exon 12. The longer form retains the second spacer region and the C-terminal transmembrane region (Fig. 3). It is not yet known whether a given cell class produces both proteins. The marked similarity between QSOX1 and QSOX2 in several highly conserved regions suggests that both paralogs may be recognized by some polyclonal antibodies raised against the entire protein. However the sequences selected for two peptide antibodies make them likely specific for QSOX1 (14) and QSOX2 (98). Where it appears prudent, we will refer to the distribution of QSOX immunoreactivity without specifying which form of the enzyme is being recognized. We note that in most tissues QSOX1 is considerably more abundant than QSOX2 (see later).

In humans, QSOX1 has been found associated with the islets of Langerhans in the pancreas, the parotid gland, apocrine glands of the skin, and cells in the small intestine that are known to secrete peptides and proteins (83, 87). In addition, immunohistochemistry showed high levels of expression in many other tissues with a heavy secretory load including the pituitary, plasma cells, and prostate (87). QSOX has been found by immunolocalization in a large number of structures in rat and guinea pig brains (1). Neurons that generate disulfide-containing neuropeptides express QSOX strongly (59, 60). QSOX may be involved in neuron guidance, settling, and maturation (61). Tury *et al.* (88) located QSOX in the rat adenohypophysis and demonstrated that QSOX levels were regulated by estradiol. This is similar to the regulation of the guinea pig QSOX1, originally named GEC-3 (65). QSOX1 has been recognized by cDNA microarrays as an estrogen-repressed gene (42) and suggested as a candidate in the maturation of disulfide-bridged breast cancer resistance protein (BRCP, (41)).

Expression of QSOX mRNAs

Both traditional methods of observing gene expression and high throughput approaches show similar patterns. Both QSOX1 and QSOX2 have been detected in a large number of molecular profiling studies. For example, the distributions of QSOX1 and 2 in selected human tissues taken from the SymAtlas database (80) are shown in Figure 6. As noted earlier, QSOX1 appears both generally more abundant and of more widely varying distribution than QSOX2. Of the data shown, QSOX1 is particularly evident in lung, placenta, testis, and trachea. QSOX2 appears to be expressed at a higher level than QSOX1 in bone marrow and several brain tissues (amounting to a two-fold difference in whole brain; Fig. 6).

A number of other indications of the level of expression of QSOX in cell lines and tissues are available on the Web. One of the most interesting sites records the distribution of QSOX1 (QSCN6) in 60 tumor cell lines (72). QSOX1 was most highly expressed in non-small cell lung cancer tumors and brain tumors, while the lowest levels were in leukemias and colon tumors. Correlations between the expression pattern of QSOX1 and other proteins in this dataset provide interesting insights into likely physiological roles (see later).

Using Serial Access for Gene Expression (SAGE, (52)), QSOX1 and QSOX2 levels were assessed in 133 tissues. QSOX1 was at its highest level in normal tissue in the placenta, ovary, lung, and prostate, while QSOX2 was highest in the retina, pancreas, and lymph node. For both genes the greatest abundance were observed in tumors: chondrosarcomas for QSOX1 and brain tumors for QSOX2. The overall level of QSOX1 was four-fold higher than for QSOX2 over all tissues sampled.

The large number of ESTs submitted to the EST database (Unigene, Expression Profile) provides a useful alternative for assessing gene expression. For humans there are more than 3.3 million ESTs with a specific anatomical basis (Unigene Build#176). Overall, QSOX1 was 13-fold more abundant than QSOX2 (represented at rates of 295 and 22 per million ESTs, respectively). In terms of tissue distribution, Figure 7 shows that human QSOX1 ESTs are the most highly represented in lung, followed by stomach, placenta, and bone. By contrast, QSOX2 was most often found in the spleen, eye, and lymph node. By EST analysis, only in the lymph node and the spleen was QSOX2 more abundant than QSOX1.

Finally, the first expression data from invertebrate homologs have been reported for the *Drosophila melanogaster* QSOX gene CG17843 (DmQSOX2; (83)) which was found expressed at high levels in the male accessory gland. Expression was regulated by action of sexual hierarchy due to the presence or absence of the DSX^F factor (3). Interestingly, in RNAi experiments for the three *C. elegans* homologs (83), there was no embryonic or maternal lethality, however for F47B7.2 (CeQSOX3, (83)) RNAi caused slow growth, sluggishness, or uncoordinated movements in two of three experi-

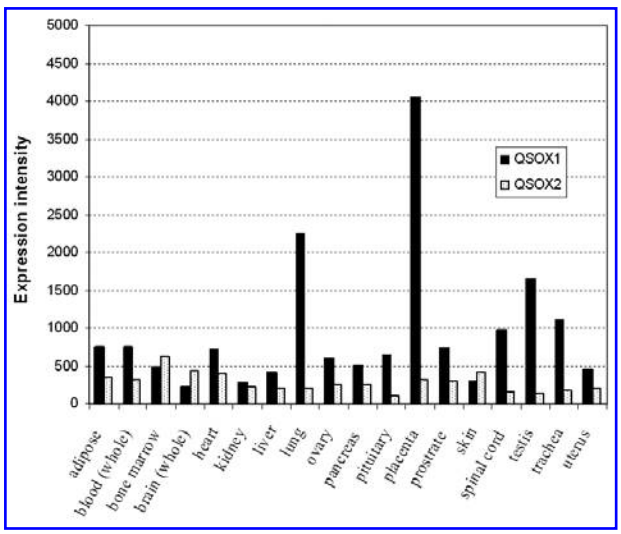


FIG. 6. Expression profiling for QSOX1 and QSOX2 transcripts in selected human tissues. The abundance was taken from the GNF SymAtlas expression database (80) queried for QSCN6 and QSCN6L1.

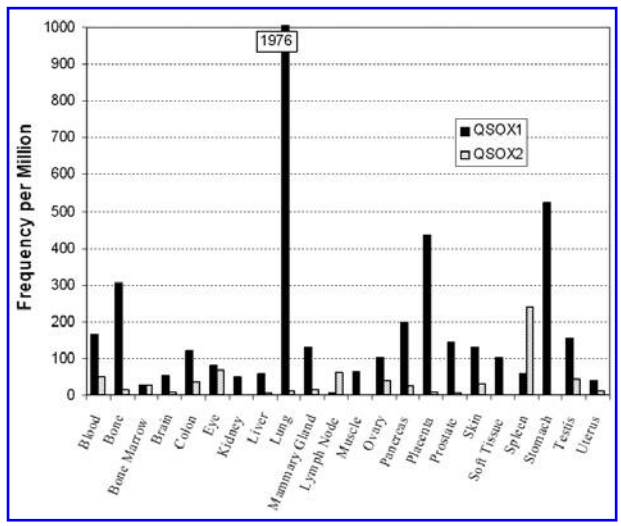


FIG. 7. Frequency of QSOX1 and QSOX2 ESTs in 27 human tissues. Data were taken from Unigene Build #176. As shown by other approaches, QSOX1 was highest in most tissues, especially the lung, stomach, placenta, and bone, while QSOX2 was highest in the spleen, eye, lymph node, and blood.

ments reported in Wormbase. For the other two paralogs, there were no reported phenotypes.

Intracellular location of QSOX

Early reports showed that QSOX protein can be found extracellularly, for example, in seminal fluids (6, 67), egg white (36, 37), and quiescent fibroblast supernatants (14). However, QSOX is also found intracellularly and it will be interesting to learn the relative significance of these diverse locales. Thus QSOX has been located in ER (83, 88), golgi (59, 83, 88), secretory granules (59, 88), and dense core granules of the pituitary (88). Sulfhydryl oxidase immunoreactivity has also been reported associated with mitochondria or in the mitochondrial matrix during the development of mammalian testicular cells (4, 6, 7, 48). Finally, QSOX2 is reported to be predominantly directed to nuclear and plasma membranes (98). Overall, QSOXs show a range of cellular locales, perhaps reflecting multiple intra- and extracellular functions in diverse cell types and tissues.

PHYSIOLOGICAL ROLES OF QSOX

The facility with which QSOX1 introduces disulfides into peptides and proteins and the finding that the avian enzyme cooperates with PDI *in vitro* to rapidly generate the correct pairings in reduced RNase, leads to the concept that a major role of QSOXs is in the generation of disulfides *in vivo*. Several notes of caution should be raised here. First, the only detailed study of enzymatic specificity to date has utilized avian QSOX1. Second, there are no knock-out studies for vertebrate QSOXs, nor are there any known disease-causing mutations. Third, the pathways for the interconversion of thiols and disulfides are marked by a multiplicity of catalysts and processes with overlapping specificity. While this may severely complicate the interpretation of knockout or knockdown data, a distant goal would be to learn the percentage of protein disulfides introduced by each of the various primary vertebrate oxidases, including human ERO1-Lα, ERO1-Lβ (64), QSOX1, and QSOX2. Such percentages will likely depend both on protein substrate and on cell type.

We next present additional observations which point toward possible roles of the QSOX family. One correlation comes from an examination of the NCI 60 tumor cell line microarray data of about 8000 unique genes mentioned earlier (72). An analysis for genes whose expression most closely correlate with QSOX1 expression showed that the first and third most highly correlated proteins are collagen type IV α1, and lysyl oxidase: proteins with key roles in extracellular matrix formation. A number of the other correlates are clearly involved extracellularly (plasminogen activator, plasminogen activator receptor, integrin β1, and a CYR61-like disulfide-rich extracellular signaling protein). These observations reinforce our earlier suggestions (16, 83) that QSOX1 is likely important in ECM formation/remodeling. Indeed, QSOX1 was originally identified as a gene that was upregulated when human fibroblasts reached proliferative quiescence with the elaboration of ECM (16, 83). QSOX might be secreted, or secured at the plasma membrane surface via its C-terminal transmembrane span, to provide an oxidizing catalyst for the elaboration of extracellular structures that cannot be completed intracellularly (83).

QSOX and the generation of hydrogen peroxide

Whereas disulfide bond formation (both intra- and extracellularly) appears to be a general function of QSOX, the next two sections suggest that the collateral generation of hydrogen peroxide may be also important. For example, sulfhydryl oxidase secretion has been suggested as a contributor to antimicrobial action (67) although this interesting suggestion has not been explored experimentally. Perhaps extracellular or membrane-anchored QSOX coupled with the established (27, 79) secretion of GSH from the apical surfaces of cells might contribute to the generation of hydrogen peroxide outside the plasma membrane surface. Hydrogen peroxide, in conjunction with extracellular peroxidases (see Refs. 13 and 50), would drive antimicrobial action. The salvage of oxidized glutathione fragments by reabsorption (81) could then maintain a cycle for the continual generation of hydrogen peroxide in extracellular luminal regions. In addition to possible antimicrobial involvement, Mairet-Coello *et al.* (59) suggest that QSOX may be involved in hydrogen peroxide generation in brain and thus, conceivably, signal transduction.

Does protein disulfide bond formation contribute directly to oxidative stress?

The idea that oxidative stress is associated with protein secretion has been raised earlier in the context of ERO1p (30, 32, 86, 102), but it applies equally to QSOX catalyzed oxidative processes (59, 83). Choosing one important example: oxidative stress is believed to be a significant factor in the eventual death of beta cells during the progression to type II diabetes (e.g., reviewed in Refs. 2 and 31). Here a decreasing number of pancreatic beta cells are driven to produce ever-increasing levels of insulin. Considerable emphasis has been placed on the role of reactive oxygen species, generated by elevated glucose levels, in the eventual demise of beta cells, however, there is an additional, largely neglected, source of reactive oxygen species in beta cells: the hydrogen peroxide that is an immediate product of net insulin disulfide bond formation by flavin-dependent sulfhydryl oxidases (11, 21, 64, 83, 85). Here, the elevated hydrogen peroxide levels would be a direct consequence of hyperinsulinemia: the three disulfide bridges in each mature insulin molecule would be expected to lead to the release of three molecules of hydrogen peroxide. However, this stoichiometry may be a lower limit if iterative cycles of reduction and reoxidation intervene in securing the correct disulfide pairings (74, 86).

Both ERO1p and QSOX1 and 2 are found in islet tissue by profiling RNA expression levels (80). In terms of protein expression, immunohistochemistry shows that the islets of Langerhans stain strongly for QSOX1 (83) and that this expression is markedly upregulated with the hypersecretion of insulin that is associated with insulinomas (Turi and Coppock, unpublished; (87)). Whether sulfhydryl oxidases are upregulated during the progression to type II diabetes remains to be established.

QSOX2 and apoptosis

In an important study, Schwab and co-workers have recently identified the second human QSOX (SOXN, QSOX2),

through its role in promoting γ-inteferon induced apoptosis in neuroblastoma cell lines (98). SOXN is described as "a major player" in retaining the sensitivity of these cells to apopotosis and "an important link in the maintenance of drug-therapy-induced apoptosis".

METAL-DEPENDENT SULFHYDRYL OXIDASES

The first sulfhydryl oxidase to be purified was an iron-dependent sulfhydryl oxidase found in bovine skim milk (43). It has not received a detailed enzymological dissection and there is no sequence information on this activity. Rodent skin sulfhydryl oxidase was reported to be a copper-dependent metalloenzyme (100), but its sequence subsequently showed that it was homologous to the QSOX family (10, 63). Certainly QSOX1 is abundant in the keratinocytes of skin epidermis (83, 87). There is now evidence that both oxidized and reduced forms of sulfhydryl oxidase can bind transition metal ions (10). Pure avian QSOX1 contained insignificant levels of copper until exposure to low levels of Cu^{2+} in the buffer (10). In addition to nonspecific binding, a unique metal binding site close to the flavin is created on 4-electron reduction of the enzyme. Experiments using Zn^{2+} as a redox inactive surrogate for copper confirmed the profound perturbation of the internal redox equilibrium that accompanies binding the metal in a thiol-rich environment. This coordination leads to inactivation of avian QSOX and is reversed slowly as flavin-mediated reoxidation of the CxxC motifs extrude the bound zinc ion (10). Whether these apparently adventitious metal binding sites have any physiological significance remains to be seen. In addition to the possibility of direct inhibition of enzyme activity by zinc ions, a recent report (9) shows that the administration of zinc sulfate to olfactory epithelia leads to a major decrease in QSOX expression levels. Zinc salts are the active ingredient in some nasal sprays allegedly implicated in anosmia associated with epithelial damage. It is interesting that the highest expression level of QSOX1 in the SymAtlas array database (80) for mouse is the vomeronasal gland, with the main olefactory epithelium scoring as the fourth highest expressing tissue.

ERV1, AUGMENTER OF LIVER REGENERATION AND ERV2

Overview

A number of diminutive stand-alone proteins with sulfhydryl oxidase activity have now been described that share a helix-rich FAD-binding fold utilized as the oxidase domain of QSOX. Figures 2A and 2B show that the isoalloxazine ring is bound in the mouth of a bundle of four helices with a short C-terminal fifth helix perpendicular to the main helix axis completing the flavin binding fold. A conserved CxxC motif lies on the *re* face of the flavin ring between helices 2 and 3 (29, 99). As mentioned earlier, the C-terminal cysteine of this motif is perfectly positioned to form a covalent adduct with the C-4a position of the isoalloxazine ring during transfer of reducing equivalents to the flavin.

Figure 8 shows a representation of selected members of the ERV1/ALR family aligned by the position of this conserved redox-active disulfide (54). Many of these small catalysts have an additional redox-active disulfide centers that interact with the proximal disulfide. Yeast ERV2p, for example, has an essential CxC motif within a C-terminal extension (Fig. 2A, and ScERV2p in Fig. 8; see earlier (29)). In contrast, ScERV1p has an N-terminal CxxC motif that Lisowksy and co-workers have shown is in redox communication with the proximal disulfide (33). Here a C30S mutation of ScERV1p effects a dramatic change in color of the oxidized wild-type protein from a yellow to a "black" variant whose spectrum resembles a charge-transfer complex between oxidized flavin and a thiolate donor (33). It appears likely that the C30S mutation allows C33 to form a new disulfide pairing with C130. The resulting C133 thiolate would then form a

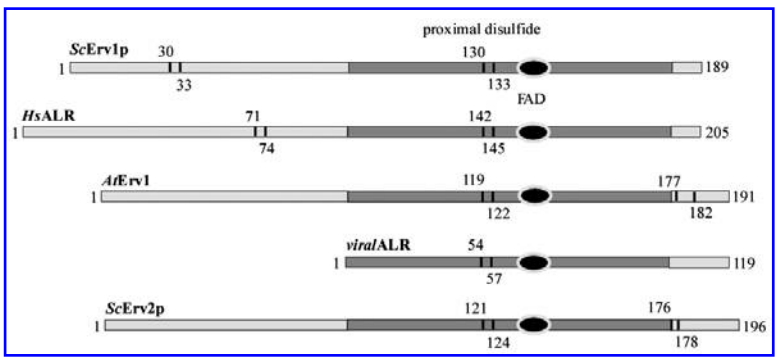


FIG. 8. Diagram of the arrangement of redox centers in selected members of the ERV1/ALR family. The protein chains are drawn approximately to scale and aligned using the position of the proximal disulfide. The *black oval* represents FAD arbitrarily placed within the central helical domain (defined by the crystal structures; *darker bar*). Only the proposed redox-active disulfides are shown. Thus the figure omits a structural disulfide (C159–176 in ScERV1p) which is conserved in HsALR (NP_005253), AtERV1 (CAD83013), ScERV1p (NP_011543), and ScERV2p (NP_015362) subunits: this disulfide is absent in the viral ALR analogues (e.g., P23373) and in all QSOXs. Also not shown are two structural disulfide bridges $C94_A$-$C204_B$ and $C94_B$-$C204_A$ linking A and B subunits at the N and C-termini of the flavin binding domain in HsALR (see also Fig. 2B). This feature is absent in ScERV1p, AtERV1, viralALR, and ScERV2p.

charge-transfer complex with the isoalloxazine ring as a prelude to the net transfer of reducing equivalents to the flavin center (see earlier). The mammalian homolog of ERV1p, ALR, also has an N-terminal CxxC feature, but this disulfide lies about 30 residues closer to the FAD binding domain (Fig. 8). In contrast, ERV2p has a catalytically essential additional redox-active C-terminal CxC motif (29, 77). Finally, a series of plant ERV1 homologs show a conserved C-terminal CxxxxC disulfide (54).

The diverse placement of this second disulfide redox center in members of the ERV1/ALR family may not indicate fundamental differences in catalytic mechanism. Thus the N-terminus and proximal disulfide/flavin of one subunit of these dimeric proteins is close to the C-terminus of the other subunit. Accordingly, a second redox-active disulfide located on a flexible extension at the C-terminus could utilize an inter-subunit disulfide exchange (as observed in ERV2p (29) and *Arabidopsis* ERV1 (54); Fig. 8). In contrast, an extra disulfide placed at the N-terminus (e.g., in ScERV1p and HsALR) could employ an intra-subunit electron transfer pathway. Determination of the structures of these full length proteins should provide critical insight towards the disposition of redox centers within these poorly understood proteins. Finally, of all the ERV1/ALR proteins examined to date, only the viral ALR homologs (76) have a single (proximal) disulfide (Fig. 8). This center presumably interacts directly with a virally-encoded glutaredoxin during the insertion of disulfides into viral envelope proteins (94).

Phylogeny of small sulfhydryl oxidases

Although a detailed examination of the representation of flavin-dependent sulfhydryl oxidases in microbial genomes is beyond the scope of this review, a number of interesting observations can be made. As mentioned earlier, baker's yeast contains three sulfhydryl oxidases ERO1p, ERV1p, and ERV2p, but no QSOX. Several other organisms lack QSOX. First, several large DNA viruses including *Vaccinia*, PBCV, and ASFV have a single ERV1p-like protein (15, 83) which Moss and coworkers have shown is used to generate envelope protein disulfides in the host cytoplasm (75). This viral enzyme is encoded by one of 49 genes that are completely conserved in pox viruses isolated from both insects and vertebrates (89). *Dictyostelium discoidans* has a single ERV1p. In contrast, the amitochondrial diplomonad *Giardia* apparently contains neither ERV1p nor ERV2p. Finally, the microsporidians are obligate intracellular parasitic fungi with vestigial mitochondria (45, 96). The human pathogen *Encephalitozoon cuniculi* has one of the smallest eukaryotic genomes and lacks ERV1p (found in the mitochondrial intermembrane space of this organelle in *S. cerevisiae*), but contains an ERV2p-like protein with the characteristic C-terminal CxC motif (Fig. 2). This sulfhydryl oxidase may contribute to the fabrication of the tubular disulfide-crosslinked protein filament that is deployed rapidly from microsporidian spores during host infection (18, 45, 93).

Substrate specificity

Yeast ERV2p (ScERV2p) is an ER-resident flavoprotein (26, 77) that interacts with PDI as one pathway to disulfide generation in yeast. While evidence for a complex between PDI1p and its cognate primary oxidases has been amassed from gel-crosslinking studies (77), it will be interesting to evaluate the detailed enzymology of this interaction. ERV2p directly oxidizes reduced lysozyme rather slowly (26). DTT is a convenient model substrate of ERV2p (k_{cat} 239/min, K_m 6.9 m*M* (77)). ERV1p, and the mammalian and plant ALR homologs, oxidize reduced lysozyme, reduced thioredoxin, and DTT with modest catalytic efficiencies (19, 33, 54). Glutathione is not a detectable substrate of these enzymes.

Mammalian ALR is an enigmatic protein with sulfhydryl oxidase activity

The remaining brief comments on these small sulfhydryl oxidases are directed at perhaps their most enigmatic member: the mammalian augmenter of liver regeneration. ALR, also known as hepatopoietin, is found in long and short variants (lacking the N-terminal extension (Fig. 8). ALR appears to have multiple roles in the regulation of cell growth and differentiation (12, 23, 47, 51, 55, 57, 69, 90). Mammalian ALR has been found in the mitochondrial intermembrane space (5, 47, 51), the cytosol (34, 58), in the nucleus (58, 92), and as a secreted growth factor (20, 23, 55, 90).

The physiological reducing substrates of ALR have yet to be identified and may depend on cellular location. Lisowsky and Lill and their colleagues suggest that mitochondrial ALR, and its yeast counterpart, are involved in the fabrication of cytoplasmic Fe/S centers (51). Does this aspect involve oxidation of thiols or Fe/S centers? A number of proteins resident in the mitochondrial intermembrane space (including thionein, TIM10 and 13, superoxide dismutase, and ALR itself) have disulfide bonds; is ALR involved in their maturation or regulation *in situ* (19, 47)?

Uncertainty concerning the physiological substrates of ALR extends to their electron acceptors *in vivo*. While flavoprotein oxidases employ molecular oxygen as their immediate electron acceptor (62), cytochrome *c* has been found to be a ~ 100-fold better substrate (in terms of k_{cat}/K_m determined *in vitro* (19)). *In vivo*, oxidized cytochrome c is much more abundant in the mitochondrial intermembrane space than dissolved oxygen, further favoring the cytochrome pathway (19). These results provide a means to couple the oxidation of ALR to the respiratory chain without the generation of hydrogen peroxide (19). Clearly we have much to learn about ALR and its multiple roles in cellular redox signaling and mammalian physiology.

CONCLUDING REMARKS

Catalysts containing the ERV1/ALR fold range from what is probably the smallest known flavoenzyme (the viral ALR) to much larger multidomain proteins found in all multicellular organisms. Members of this family serve as extracellular growth factors, participate in intracellular and extracellular signaling networks, are involved in the assembly of Fe/S centers, have been implicated as a component of antimicrobial defense and, of course, have been repeatedly suggested as contributors to the net generation of cellular disulfides.

Most biochemists are unaware that mechanisms for the net generation of disulfides in metazoan, protist, and plant systems remain poorly understood. While the flavin-linked

sulfhydryl oxidases, including, for example, mammalian ERO1-Lα and -Lβ (11, 64, 68) and the QSOX members discussed here, are likely major contributors, there may be alternate routes whose significance has yet to be appreciated. As noted, we cannot yet identify the relative importance of these almost ubiquitous QSOX enzymes in the secretion of any particular protein in any given cell type. Although we have mentioned suggestive correlations, evidence from mammalian knockout/down studies is lacking. Further, such attempts to dissect oxidative protein folding will usually face complications due to the diversity and degeneracy of thiol/disulfide exchange pathways—an inherent consequence of the impressive nucleophility of the thiolate anion.

In vitro, avian QSOX1 shows a prodigious, if rather unmodulated, ability to insert disulfides into conformationally mobile or unfolded reduced proteins (38). Perhaps because it is a fusion of an oxidizing ERV1/ALR module and thioredoxin-like domains, avian QSOX does not necessarily require the presence of separate PDI-like proteins to communicate with substrate proteins undergoing oxidative folding. QSOX1 does not appear to have significant intrinsic protein disulfide isomerase activity (D. Cripps and C. Thorpe; unpublished observations), but can cooperate with exogenous PDI to secure the correct pairings in reduced RNase (38). The interplay and staging between these two catalysts has yet to be elucidated, even *in vitro*. Given the paramount importance of PDI and PDI-like proteins in oxidative protein folding, an important ongoing challenge is to identify the enzymological and structural aspects of their interaction with presumed cognate primary flavoprotein oxidases within the lumen of the ER.

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ABBREVIATIONS

ALR, augmenter of liver regeneration or hepatopoietin; ER, endoplasmic reticulum; ECM, extracellular matrix; ERV1p and ERV2p, proteins originally termed essential for respiration and vegetative growth; PDI, protein disulfide isomerase; QSOX, Quiescin-sulfhydryl oxidase; SAGE, Serial Access for Gene Expression; TRX, thioredoxin.

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