Generating Disulfides in Multicellular Organisms: Emerging Roles for a New Flavoprotein Family*

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Peptides and proteins destined for secretion in multicellular organisms usually contain disulfide bonds, from small peptides to massive extracellular matrix (ECM)2 proteins with hundreds of disulfide bridges. Disulfides are important to the structure, stability, and regulation of many proteins having at least one extracellular domain; they are critical to the formation and remodeling of the ECM and other disulfide networks, and they are crucial elements in various redox signaling pathways. However, the pathways for their biosynthesis in multicellular organisms remain surprisingly cryptic. We do not really know how a single protein disulfide bond is introduced in any metazoan, green plant, or protist.

Why is our understanding of oxidative folding in so rudimentary a state? One reason is the very reactivity of thiolate nucleophiles and the degeneracy of pathways for the interconversion of thiols and disulfides. A second factor is the facile non-enzymatic oxidation of thiols by a number of potential cellular oxidants including GSSG (1). A third issue is the common misperception that oxygen is a facile oxidant of juxtaposed thiols, a reaction that is spin-forbidden and strongly catalyzed by traces of redox-active transition metal ions (notably copper and iron). Finally, multicellular organisms have additional pathways for disulfide bond formation that are not shared with the genetically tractable yeast systems.

Scope

A key issue in this Minireview is the identity of the oxidizing catalysts for disulfide bond formation in multicellular organisms. Although we identify likely candidates, it is important to recognize that there may be major routes to disulfide generation that remain to be uncovered. A second issue is the involvement of the protein disulfide isomerasers (PDIs) (for representative reviews see Refs. 2–5) in addressing incorrectly paired cysteine partners, and the phasing of PDI’s cooperation with the other components needed for the successful exit of a mature protein from the quality control system of the ER (6, 7). Again, the precise roles of PDIs in this critical aspect of oxidative folding are still uncertain, in part because of the difficulties inherent with systems in which thiols and disulfides are in complex and rapid flux. Although many aspects of these fascinating proteins remain to be resolved, there is one feature of PDI that can be addressed definitively. PDIs are not “oxidases,” and they are not enzymes showing “oxidase activity.” Oxidases, according to the Enzyme Commission, use the electrons abstracted from one substrate to reduce molecular oxygen. PDIs, in their oxidoreductase mode, just exchange one disulfide for another, thereby shifting the burden of the disposal of pairs of reducing equivalents elsewhere. Calling PDI an “oxidase” tends to divert attention from this critical aspect of oxidative folding: what to do with the pairs of electrons liberated with every disulfide made. Sulphydryl oxidases accomplish this task with the stoichiometry:

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2R–SH + O_2 \rightarrow R–S–S–R + H_2O_2.
\]

This essentially irreversible reaction can only proceed rapidly using a cofactor that can communicate facilely both with thiols and molecular oxygen. In eukaryotes the best understood of these sulphydryl oxidases are flavin-linked.

Perspectives: Making Protein Disulfide Bonds in Prokaryotes and Yeast

An important perspective for all oxidative folding comes from investigations on the Escherichia coli periplasm (for example, see Refs. 8–12). These studies have identified the net oxidant for disulfide bond insertion, the requirement for an isomerase system to correct disulfide connectivities, and the challenge of minimizing futile cycles between multiple components carrying redox-active dithiols. Ultimately, oxidative folding is driven by an integral membrane protein, DsbB, that relays reducing equivalents to coenzyme Q and thence to the respiratory chain, neatly avoiding the need to interact with oxygen directly (Fig. 1A). Emerging studies on pathways for generating disulfide bonds in the mitochondrial intermembrane space, a compartment with distant evolutionary lineage to the bacterial periplasm, suggest interesting parallels (13).

Disulfide bond generation pathways in the yeast ER appear entirely different. Here, the generally accepted view is depicted in Fig. 1B. For simplicity the arrows depict a unidirectional flow of pairs of reducing equivalents toward molecular oxygen. A disulfide bond is inserted into the folding substrate with reduction of a CXXC disulfide in a PDI. (There are multiple PDI or PDI-like proteins in all eukaryotes (3–5, 14), and the one here is suggested to be PDI1p.) Reduced PDI is then believed to be the immediate substrate for either of two FAD-dependent oxidases, Ero1p (15, 16) or Erv2p (17–19), prior to the final reduction of oxidants carrying redox-active dithiols. Ultimately, oxidative folding is driven by an integral membrane protein, DsbB, that relays reducing equivalents to coenzyme Q and thence to the respiratory chain, neatly avoiding the need to interact with oxygen directly (Fig. 1A). Emerging studies on pathways for generating disulfide bonds in the mitochondrial intermembrane space, a compartment with distant evolutionary lineage to the bacterial periplasm, suggest interesting parallels (13).

1 The abbreviations used are: ECM, extracellular matrix; ALR, augmenter of liver regeneration; ER, endoplasmic reticulum; PDI, protein disulfide isomerase; QSOX, quiescin-sulphydryl oxidase.

2 The on-line version of this Minireview (available at http://www.jbc.org) contains supplementary teaching material.

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This is illustrated in Fig. 2 for Erv2p (17). The penultimate arrow involves 2-electron reduction of the flavin cofactor by a pair of thiols: one (C57) poised to interact with the cofactor directly and the other (C54) positioned to reform a disulfide bond as the pair of electrons is transferred to the flavin. The final catalytic step is the reoxidation of reduced cofactor to generate hydrogen peroxide (see below).

The QSOX Family of Sulfhydryl Oxidases

Before Erv1p or Erv2p were discovered to be flavin-dependent sulfhydryl oxidases, a larger protein incorporating an Erv-like domain, first called Quiescin Q6 (20), was found to be a sulfhydryl oxidase (21). The substrate specificity has been best characterized for a sulfhydryl oxidase isolated from avian egg white. Although the enzyme oxidizes small thiols like GSH, unfolded reduced proteins are much better substrates (22). Here, typical $k_{\text{cat}}$ values are $\sim 1000$ protein disulfides introduced per min with $K_m$ values of $\sim 150 \mu M$ (on a per $-SH$ basis). This direct and facile oxidation of a seemingly unlimited array of reduced unfolded proteins and peptides (22) is in clear distinction to the yeast and human Ero oxidases and yeast Erv2p. These oxidases rely on PDI to mediate the flow of reducing equivalents between client reduced proteins and molecular oxygen (Fig. 1B) (18, 23–25).

Sequencing of the avian egg white (21) and the rat seminal vesicle sulfhydryl oxidases (26) showed that they were founding members of a new family of multidomain sulfhydryl oxidases (schematically depicted in Fig. 3). These QSOXs are found in all metazoans and in those plants and protists for which genomes have been sequenced (21, 26–28). In humans the sequences have been frequently annotated as PDI-like or given names that are suggestive of biological significance such as “cell growth-inhibiting factor” or “bone-derived growth factor” (26–28).

Humans have two QSOX paralogs QSOX1 (QSCN6) and QSOX2 (QSCN6L1, SOXN) showing about 35% identity over 740 amino acids (27–29).

QSOX Domain Structure and Mechanism

In metazoans, two thioredoxin domains follow a typical N-terminal signal sequence (Fig. 3). The first thioredoxin domain (Trx1) has a WCXHC motif typical of many PDIs whereas Trx2 appears redox-inactive. Plants and protists lack the second thioredoxin domain but retain a highly helical “spacer” module fused to the Erv/ALR flavin-binding domain (27). The C-terminal region varies widely among species but invariably ends with a single transmembrane span that can serve as a membrane anchor (27). This C-terminal region is frequently spliced out, leading to the most common short form of the enzyme (604 amino acids for the human enzyme). The longer form has been detected in brain (30). Conventional ER retention sequences are absent in QSOXs, yet the enzyme is observed in the ER (see later).

Fig. 2 provides important insight into the likely flow of reducing equivalents within the Erv/ALR domain of QSOX (17). What, then, are the roles of the thioredoxin domains in catalysis? Partial proteolysis of the avian egg white QSOX (31) and site-directed mutagenesis of human QSOX13 show that the first CXXC disulfide is critical for effective catalysis of protein oxidation and is the site of entry of reducing equivalents. Hence the general flow of reducing equivalents in QSOX1 is clear as shown below.

Reduced client protein $\rightarrow$ Trx1 domain $\rightarrow$ Erv/ALR domain $\rightarrow$ oxygen

This ancient fusion of thioredoxin and Erv/ALR domains (21, 26, 32) provides an important catalytic advantage over an isolated Erv domain in the oxidation of protein substrates (31). Although one might assume that the single domain flavoprotein Erv2p was an evolutionary precursor to QSOX, the phylogeny seems to point to the opposite conclusion. Thus yeast/fungi have Erv2p, but not QSOX, although these organisms appar-

3 E. Heckler and C. Thorpe, unpublished observations.
secretory load or those that accumulate protein disulfides intracellularly as a result of terminal differentiation (27, 28, 34).

**QSOX Location and Possible Physiological Roles**

Human QSOX1 appears generally more abundant than either QSOX2 or Erv1-α and -β in a range of expression profiling, serial analysis of gene expression, and expressed sequence tag frequency compilations (27). Expression of QSOX1 is especially prominent in differentiated tissues generating high levels of disulfide-containing proteins. For example, Fig. 4 (A–C) shows human epidermis, sebaceous gland, and hair follicle. QSOX1 is also abundant in the seminal vesicle (Fig. 4D), in the syncytiotrophoblastic layer of the placenta (Fig. 4E), and the eccrine gland (Fig. 4F). In addition, plasma cells that express high levels of immunoglobulins consistently show strong QSOX1 staining (for an example see Fig. 7D in Ref. 28). Detailed analyses of the distribution of QSOX1 in the developing rat brain (35) and in rat peripheral tissues (34) have appeared.

QSOX1 was initially identified as a protein secreted from quiescent human fibroblasts (20) and is present in chicken egg white (36), mammalian seminal fluid (26, 34, 37), Chinese hamster ovary epithelial cell supernatants (26), and blood serum (38). However, QSOXs have also been found in the ER, Golgi, and secretory granules and are located at the cell surface (27–29, 35, 39). The long form of QSOX, with its transmembrane span, may secure the oxidase at the plasma membrane surface (29, 30). In considering potential redox roles for surface-bound QSOX, there is now considerable evidence for functionally important thioldisulfide interconversions at the outer face of the plasma membrane. These often involve reductions mediated by protein disulfide isomerases (for example see Refs. 40–43), and so these effects could be moderated or reversed by surface sulphydryl oxidases.

The abundance of QSOX1 in bone, the correlations suggesting coordinate expression of QSOX with certain collagens and other extracellular matrix components (27, 28), and emerging data from *Caenorhabditis elegans* QSOX deletion mutants (27, 44) are suggestive of roles for these oxidases in the maturation of collagen networks. QSOXs may also be important in the elaboration of disulfide-rich materials such as those involving keratin-associated proteins (in some cases comprising >33% cysteine; for example Fig. 4, A and C).

In addition to roles in the biosynthesis of structural disulfides, secreted or membrane-bound QSOX enzymes may generate hydrogen peroxide in the extracellular space for antimicrobial effects (26, 27, 37) and for cellular signaling (27, 45). Finally, QSOX2 expression in neuroblastoma cells is a key step in maintaining their apoptotic response to cytostatic drugs (29).

**The Interaction between Flavin-dependent Oxidases and PDIs**

The pioneering work of the Fass, Kaiser, Lisowsky, and Weissman laboratories on yeast Erv2p and Ero1p and the
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A rapid accumulation of oxidized PDI would certainly favor oxidative folding in a model system (22, 27, 28). Here QSOX works first and PDI functions only slowly (22).4 Here QSOX works first and PDI functions only slowly (22).

Logical additional features might include the extreme macro-molecular crowding within the lumen of the ER and the possibility of transitory multienzyme complexes promoting disulfide bond formation and isomerization.

However, an efficient, unregulated oxidation of reduced PDIs by these flavoprotein oxidases would be highly damaging for the cell. In the mammalian ER, PDI is largely reduced and present at a concentration approaching millimolar (4, 47–49). A rapid accumulation of oxidized PDI would certainly favor protein disulfide bond formation but deprive the ER of reduced PDI that is essential for the isomerization of incorrectly placed protein disulfide bond formation.

Further, the subsequent reduction of PDI by GSH could promote a futile cycle that depletes cellular reductants and leads to the accumulation of hydrogen peroxide (Fig. 5). A facile flavin-linked oxidation of reduced PDI also appears unnecessary on thermodynamic grounds. PDIs from both yeast and mammals are quite oxidizing ($E^\circ$ from about −150 to −190 mV (4, 49)). Consequently a 1:5 ratio of PDI$_{ox}$/PDI$_{red}$ is sufficient to introduce typical structural disulfide bonds during oxidative protein folding in the ER (for an informative discussion of disulfide redox potentials see Ref. 50).

However, PDI may not be the direct oxidant for all proteins in the ER. For example QSOX introduces disulfide bonds rapidly into reduced client proteins but oxidizes reduced PDI very slowly (22). Here QSOX works first and PDI functions only after disulfide bonds begin to accumulate in client proteins (Fig. 5). Only low concentrations of QSOX are needed in vitro to match the modest catalytic activity of PDI (22). Although this kinetic cooperation between QSOX and PDI results in efficient oxidative folding in a model system (22, 27, 28) it remains to be demonstrated in vivo (see below). Finally it should be reiterated that we do not know the fraction of disulfide bonds that are inserted into client proteins as a direct consequence of interactions with oxidized PDI compared with alternative pathways (for example such as Fig. 6). The utilization of a particular sulfhydryl oxidase is likely to be strongly dependent on cell type, cellular locale, and client protein type.

Sulfhydryl Oxidases and Oxidative Stress

The recent finding that Ero1p, as well as QSOX (36, 37), generates one hydrogen peroxide molecule for every disulfide bond formed (51) reinforces the observation that oxidative folding entails oxidative stress (1, 7, 27, 52, 53). Professional secretory cells can export prodigious amounts of disulfide-containing proteins and thus may be particularly prone to ER stress (7, 52). A particularly interesting case involves type 2 diabetes. Here the dwindling numbers of beta cells must bear an ever increasing secretory load. Although oxidative stress is frequently discussed as a contributory factor to type 2 diabetes (for example see Ref. 54), there has been little general recognition that insulin secretion and hydrogen peroxide production are intimately coupled (7, 52, 53).

Metalloenzyme Sulfhydryl Oxidases

The term “sulfhydryl oxidase” was first coined (55) to describe an activity in bovine milk that has been extensively studied by Swaisgood and co-workers (56, 57). Milk sulfhydryl oxidase is reported to be an iron-dependent enzyme capable of oxidation of a range of thiols from glutathione to reduced chymotrypsinogen (57). Copper-containing oxidases have been described from skin (58), from rat intestinal epithelial cells (59), and from lymphocytes generating immunoglobulin M pentamers (60). All these metal-dependent enzymes remain poorly understood decades after their initial description; they lack protein sequences, metal spectroscopy, and structural information. One of them appears to be artifactual and likely reflects the adventitious binding of redox-active transition metals to multiple CXXC motifs (61). Overall, these metalloenzyme sulfhydryl oxidases deserve renewed inquiry.

Envoi

Despite the influential model studies of Anfinsen, Creighton, Scheraga, and others, we still do not understand fundamental aspects of the insertion and isomerization of disulfide bonds in eukaryotes. The recent findings of new sulfhydryl oxidases and PDI-like proteins, together with growing interest in the biochemistry of the ER, are helping illuminate the enigma that is oxidative protein folding.

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4 P. Rancy and C. Thorpe, unpublished observations.
