Disulfide relays between and within proteins: the Ero1p structure

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The essential flavoenzyme Ero1p both creates *de novo* disulfide bonds and transfers these disulfides to the folding catalyst protein disulfide isomerase (PDI). The recently solved crystal structure of Ero1p, in combination with previous biochemical, genetic and structural data, provides insight into the mechanism by which Ero1p accomplishes these tasks. A comparison of Ero1p with the smaller flavoenzyme Erv2p highlights important structural elements that are shared by these flavin adenine dinucleotide (FAD)-binding sulfhydryl oxidases and suggests some general themes that might be common to proteins that generate disulfide bonds.

A disulfide bond is nothing more than a covalent linkage between the sulfur atoms of two cysteine residues. Yet this bond is often essential to the structural stability of the protein in which it is found. Because many proteins require disulfides for correct folding and functioning, the mechanisms by which disulfide bond formation occurs are of considerable interest. Both prokaryotes and eukaryotes possess specialized cellular compartments – complete with enzymes devoted to catalyzing disulfide formation and rearrangement – in which disulfide bonds are formed [1].

In eukaryotes, oxidative protein folding occurs in the endoplasmic reticulum (ER). There, protein disulfide isomerase (PDI) catalyzes the formation of disulfide bonds in substrate proteins. To oxidize thiol groups to disulfides, the active-site cysteines of PDI must be maintained in oxidized form. In 1998, the Kaiser [2] and Weissman [3] groups independently identified Ero1p as the primary source of oxidizing equivalents for PDI. The recent publication of the crystal structure of Ero1p represents a major advance in our understanding of this important enzyme [4] and, as we describe here, the mechanism of Ero1p is beginning to be elucidated.

Oxidation of PDI involves a disulfide relay between proteins

Ero1p is a membrane-associated flavoenzyme that generates disulfide bonds and transfers them to PDI [2,3]. Both *PDI1* and *ERO1* are essential genes that are largely conserved among eukaryotes [5]. Early work showed that the essential functions of PDI and Ero1p are not identical: the thiol oxidant diamide can restore viability to an *ERO1* deletion mutant but not to a *PDI1* deletion mutant [2]. Subsequent studies showed that Ero1p directly relays disulfide bonds to PDI, which can then oxidize substrate proteins. In the absence of Ero1p, both PDI and substrate proteins are found in a reduced form [6].

How does Ero1p create disulfide bonds? The early hypothesis that oxidized glutathione, the principal redox buffer of a cell, might provide oxidizing equivalents for disulfide formation proved to be incorrect [2,7]. The prokaryotic thiol oxidation system, DsbB–DsbA, which is functionally analogous to Ero1p–PDI, uses quinone cofactors to link protein oxidation to the respiratory chain, in a pathway in which molecular oxygen is often the final electron acceptor [8]. By contrast, ubiquinone and heme, which are both components of cellular respiration, are not directly involved in disulfide production by Ero1p. Surprisingly, Ero1p creates disulfide bonds via the cofactor flavin adenine dinucleotide (FAD) [9]. Under aerobic conditions molecular oxygen oxidizes Ero1p; electron acceptors for Ero1p under anaerobic conditions are unknown [10].

A disulfide relay within Ero1p?

The structure of Ero1p is of particular interest because the Ero1p amino acid sequence does not align with any other protein of known function [2]. The overall fold of Ero1p is novel and so far has no structural neighbors [4]. Ero1p is a single domain protein that is predominantly α-helical, with five short β-sheets and two poorly structured extended loops (Figure 1).

The functional roles of the seven conserved cysteine residues of Ero1p have been examined by site-directed mutagenesis [11]. This work suggests that there are at least two disulfide bonds in Ero1p and that each has a unique role: Cys352 and Cys355 are essential to maintain Ero1p in a fully oxidized form, pointing to their central role in the *de novo* formation of disulfide bonds, whereas Cys100 and Cys105 are probably involved in interactions with PDI, because a Cys100Ala mutation abolishes the ability of Ero1p to oxidize PDI *in vivo* [11]. Consistent with this, the crystal structure shows that the disulfide pairings are Cys352–Cys355, Cys100–Cys105 and Cys90–Cys349, and that Cys208 in is an unpaired thiol form. This disulfide connectivity corroborates the model suggested by Frand and Kaiser [11] in which electrons are

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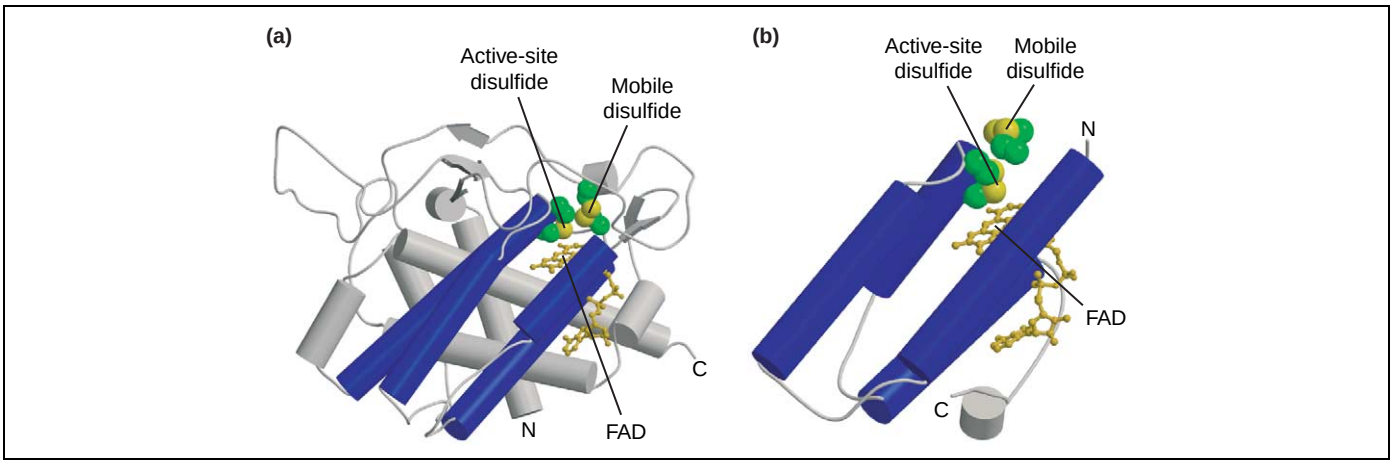

Figure 1. The 3D structure of Ero1p from *Saccharomyces cerevisiae* reveals a largely helical molecule with marked structural similarities to the Erv2p monomer. **(a)** Structure of Ero1p, with the four helices corresponding to Erv2p colored blue. 'Active-site disulfide' identifies the Cys352–Cys355 disulfide, whereas 'mobile disulfide' identifies the Cys100–Cys105 disulfide. **(b)** The Erv2p protomer. In Erv2p, the mobile disulfide comes from the second subunit of the Erv2p dimer (not shown). Here, the position of the mobile disulfide relative to the active-site disulfide is shown. Abbreviation: FAD, flavin adenine dinucleotide.

transferred from PDI to Cys100–Cys105 of Ero1p, and then within Ero1p to Cys352–Cys355.

Further crystallographic evidence supports this intramolecular disulfide relay. Cys100 and Cys105 are positioned on a flexible loop that can change its conformation by at least 17 Å. A slight modification of the protein backbone in this region would position Cys105 within disulfide-bonding distance of Cys352, which is a requirement for dithiol–disulfide exchange. The Cys352–Cys355 disulfide is positioned on a more rigid part of Ero1p, adjacent to the FAD cofactor, which probably enables Cys352–Cys355 to be readily reoxidized. Thus, electrons could be transferred from PDI to the Cys100–Cys105 disulfide, then to the Cys352–Cys355 disulfide, and then to FAD.

Erv2p and Ero1p: common structural themes

Recently, Erv2p, a second membrane-associated flavoprotein that can directly oxidize PDI, has been identified and its crystal structure solved [12,13]. Overexpression of Erv2p restores cell viability in the absence of functional Ero1p; however, deletion of *ERV2* is not lethal [14]. Erv2p is structurally related to the well-studied QSOX family of sulfhydryl oxidases, which also use FAD to drive the formation of disulfide bonds [15]. QSOX enzymes consist of a fused Erv2p-like domain, which catalyzes the *de novo* formation of disulfide bonds, and a thioredoxin-like domain (similar to those in PDI), which catalyzes oxidative folding in substrate proteins; thus, their enzymatic mechanism might well be relevant to both Erv2p and Ero1p [16]. Because Ero1p and Erv2p are the only proteins known to oxidize PDI directly *in vivo*, the crystal structures of Ero1p and Erv2p facilitate an intriguing comparison of structural elements that might be required for interaction with, and oxidation of, PDI.

The structure of Erv2p revealed a new fold for a flavoenzyme: it is a small, largely α-helical dimer belonging to the 'all-α' class of folds [13] (Figure 1). Like Ero1p, Erv2p contains a tightly bound FAD cofactor adjacent to a Cys-Xaa-Xaa-Cys motif and a second cysteine pair located on a flexible loop, although in Erv2p the mobile cysteine pair is located on the subunit opposite the Cys-Xaa-Xaa-Cys motif. Ero1p and Erv2p also share a very similar and unusual 'bent' conformation of the FAD cofactor.

Finally, the Erv2p protomer, which consists of a four-helix bundle (α1–α4) surrounding the FAD cofactor, can be superimposed onto helices α2, α7, α8 and α3 of Ero1p with a root-mean-square deviation of 1.9 Å for Cα atoms. Strikingly, although these helix bundles are similar in tertiary structure, their sequence connectivity differs. The fact that these structural elements are functionally conserved without being identical suggests that they might be vital to the shared function of the two proteins, the *de novo* formation of disulfide bonds and the oxidation of PDI.

There is a conspicuous difference between Erv2p and Ero1p – namely, the accessibility of a small-molecule electron acceptor to the active-site FAD. Erv2p contains a short hydrophobic channel that is the correct size to enable an O_2 molecule to reach the active site [13]. In Ero1p, however, FAD is buried and there is no obvious pathway by which a small molecule could contact it. This structural difference is consistent with the observation that Erv2p uses primarily O_2 as its final electron acceptor, whereas Ero1p must almost certainly use other electron acceptors (because *ERO1* is essential under anaerobic conditions) [4]. This observation raises the issues of what these final electron acceptors might be, and how they are able to interact with the buried FAD molecule in Ero1p. The final steps of electron transfer in Ero1p remain to be elucidated in more detail.

Concluding remarks: commonalities among disulfide-generating proteins

The prokaryotic DsbB–DsbA system shares obvious similarities with the eukaryotic Ero1p–PDI and Erv2p–PDI thiol oxidase pathways (Figure 2). First, Erv2p and Ero1p retain their signal sequences and thus are kept tethered to the ER membrane, and DsbB is found in the *Escherichia coli* inner membrane [17]. As Tu and Weissman [5] suggest, the localization of these essential folding catalysts to the site of initial protein translocation might

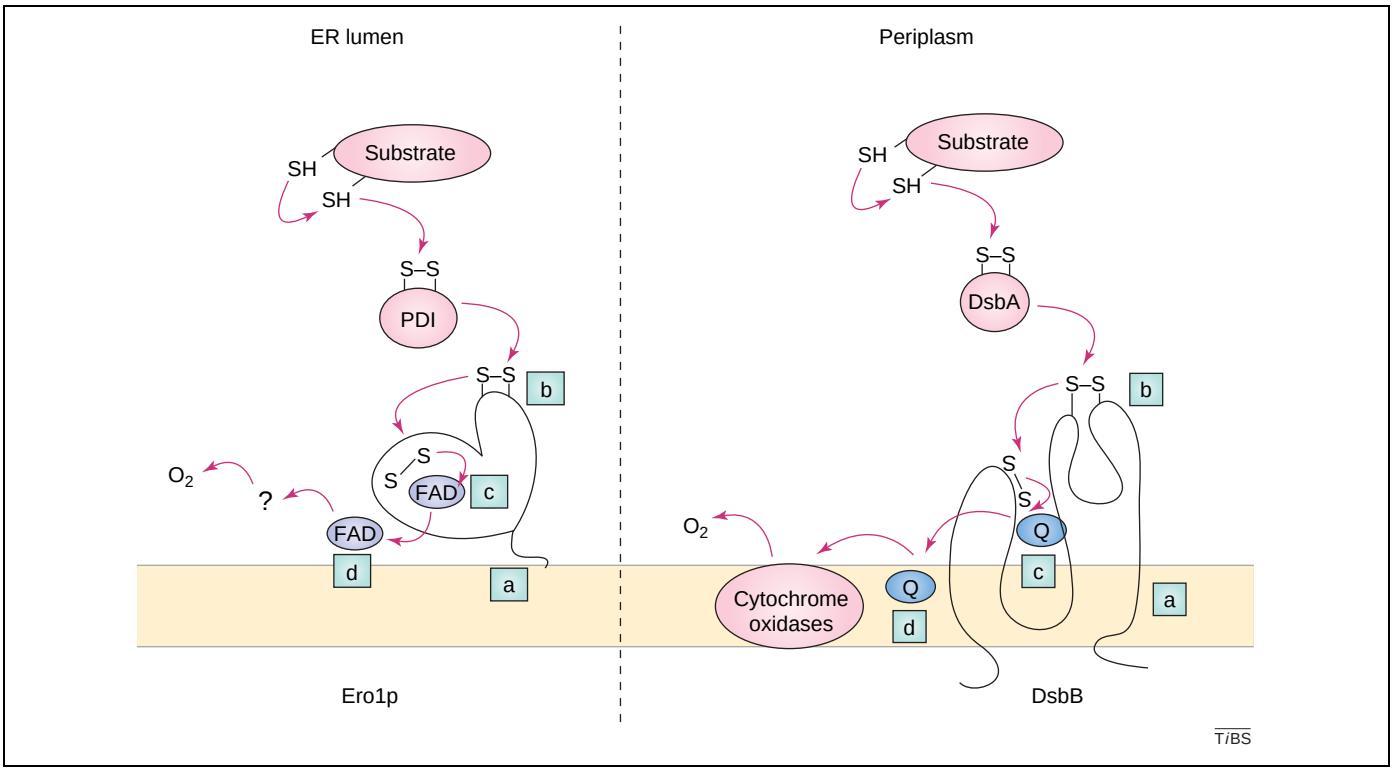

Figure 2. Model of electron transfer in Ero1p and DsbB. Red arrows indicate the putative pathway of electron transfer. Possible similarities between Ero1p and DsbB include: **(a)** their membrane localization; **(b)** a disulfide bond, probably situated on a flexible region of Ero1p or DsbB, that can transfer disulfide bonds from protein disulfide isomerase (PDI) or DsbA to a second disulfide within Ero1p or DsbB, respectively; **(c)** a disulfide bond situated adjacent to an oxidizing cofactor: flavin adenine dinucloetide (FAD) for Ero1p, quinone (Q) for DsbB; and **(d)** a tightly bound cofactor that might be able to transfer electrons to an unbound cofactor. These electrons are eventually transferred to terminal electron acceptors such as molecular oxygen.

promote productive folding by enabling substrates to fold cotranslationally.

Second, Erv2p and Ero1p function by the internal transfer of disulfide bonds from a rigid Cys-Xaa-Xaa-Cys motif adjacent to an oxidizing FAD cofactor to a disulfide pair located on a flexible loop region; this loop region then transfers disulfides to PDI, which contains four thioredoxin-like domains. Although the structure of DsbB has not been solved, a disulfide bond relay has been shown to occur from a Cys-Xaa-Xaa-Cys motif adjacent to an oxidizing quinone cofactor to a second disulfide pair, which then transfers the disulfide to thioredoxin-like DsbA [18,19].

Last, and most speculative, recent evidence points to possible similarities in the mechanism by which the cofactor might catalyze the initial formation of disulfide bonds in Ero1p and DsbB. Both proteins contain a tightly bound cofactor that could transfer electrons to unbound cofactor, which could then deliver electrons to a terminal electron acceptor. In DsbB, this transfer might occur through a quinhydrone charge-transfer complex that forms when oxidized free quinone and reduced tightly bound quinone interact [20]. *In vitro*, Ero1p catalyzes the reduction of excess free FAD, even though the active-site FAD is both tightly bound and probably buried deep within the Ero1p structure [4]. This model, in which a tightly bound cofactor is constantly reoxidized by electron transfer to a pool of free cofactor, provides a satisfying mechanism to explain how a tightly bound cofactor can be regenerated; however, much work is needed to determine the validity of this pathway.

Acknowledgements

We thank Deborah Fass and Einav Gross for supplying Figure 1. Support comes from a grant from the National Institutes of Health (GM57039 and GM64662). A.H. is a National Defense Science and Engineering Graduate (NDSEG) Fellow.

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0968-0004/$ - see front matter
doi:10.1016/j.tibs.2004.08.002

Aminoacylation of the anticodon stem by a tRNA-synthetase paralog: relic of an ancient code?

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The activation and charging of amino acids onto the acceptor stems of their cognate tRNAs are the housekeeping functions of aminoacyl-tRNA synthetases. The availability of whole genome sequences has revealed the existence of synthetase-like proteins that have other functions linked to different aspects of cell metabolism and physiology. In eubacteria, a paralog of glutamyl-tRNA synthetase, which lacks the tRNA-binding domain, was found to aminoacylate $tRNA^{Asp}$ not on the 3′-hydroxyl group of the acceptor stem but on a cyclopentene diol of the modified nucleoside queuosine present at the wobble position of anticodon loop. This modified nucleoside might be a relic of an ancient code.

Accurate protein translation requires a complete set of 20 aminoacyl-tRNA synthetases (aaRS), which catalyze the attachment of amino acids to their cognate tRNA before they are delivered to the ribosome for use in polypeptide synthesis. In each cell, there are distinct aaRS for each of the 20 amino acids found in majority of cellular proteins, although some exceptions exist [1]. Detailed structural analysis of many aaRS reveals that they are composed of at least two domains – the ubiquitous catalytic core domain and one, or a few, additional modules. The main catalytic domain is responsible for the activation of the carboxyl group of a given amino acid with ATP to form the corresponding aminoacyl-adenylates that remain associated with the enzyme, whereas the additional domain(s) is responsible, or at least contributes significantly, to the tRNA-recognition specificity and/or the efficiency of aminoacylation [1,2]. This modular design of aaRS is thought to be the result of a patchwork assembly of different functional modules during evolution [3].

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Existence of paralogs of aaRS with new functions

Exploration of fully sequenced genome has revealed the existence of 'synthetase-like' proteins, designated synthetase-paralogs, that is, proteins that most probably arose from a gene duplication followed by a speciation that retained the duplicated copies in the new species. Some synthetase-like proteins perform the correct aminoacylation of a tRNA but generate a charged species that is involved in different cellular or metabolic process than that of protein synthesis, such as biosynthesis of porphyrin, peptidoglycan or lysyl-phosphatidylglycerol (reviewed in Ref. [1]). Other synthetase-like proteins are shorter compared with the corresponding functional synthetases and are found in organisms from all three domains of life [4]. The function of many of these synthetase-like proteins remained unknown until recent work from several laboratories revealed that these enzymes are involved in a variety of metabolic processes, some being distinct from protein synthesis. For example, some paralogs contain only editing domains such as AlaX, ProX and ThrX, which deacylate mischarged tRNAs [1]. Other synthetase paralogs contain only the activation domain – one such protein is a minimalist histidyl-tRNA synthetase-like protein (denoted HisZ). This enzyme, when associated with another protein (HisG), catalyzes the ATP-dependent phosphoribosyltransferase reaction in