Disulfides out of thin air

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The sulfhydryl oxidase Erv2p generates disulfides from oxygen in an FAD-dependent process. The structure of Erv2p shows that during this reaction, a dramatic movement of the enzyme's C-terminal arm occurs.

The chemistry of disulfide exchange in biological systems is well-studied, but the origin of disulfide bonds is not. On page 61 of this issue of *Nature Structural Biology*, Gross *et al.*[1] report the structure of Erv2p, a sulfhydryl oxidase that, when supplied with oxygen and free sulfhydryls, generates disulfides *de novo*. The authors propose an elegant disulfide shuttle mechanism where a flexible C-terminal arm of Erv2p grabs a disulfide bond from its creation site and then whips it out to offer it up to other proteins. We are now starting to understand how Erv2p manages to pull disulfides out of thin air.

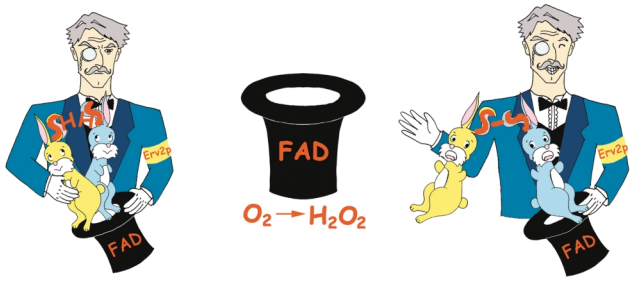

Fig. 1 Disulfides are generated from oxygen in an FAD-dependent reaction. The magician is a protein named Erv2p, the cysteines are shown as rabbits, the FAD-dependent active site of Erv2p is shown as the magician's hat. Prior to insertion into the active site, the rabbits' ears are in the reduced thiol form. The magician's C-terminal arm inserts them into the active site, oxygen is reduced to hydrogen peroxide in an FAD dependent reaction and the rabbits hop out of the active site connected by a disulfide bond. The crystal structure of Erv2p reported by Gross *et al.*[1] predicts that a dramatic 10 Å movement of the Erv2p's flexible C-terminal arm occurs during this reaction. We thank Connie Wong (Stanford) for the preparation of this figure.

Where do disulfides come from?

Disulfide bonds are vital for protein folding and stability. But until very recently it was unclear where these bonds actually come from. In eukaryotic cells, disulfide bonds are introduced into nascent peptide chains within the lumen of the endoplasmic reticulum. It has been known since the time of Anfinsen in the early 1960s that enzymes such as protein disulfide isomerase (PDI) catalyze disulfide transfer reactions[2]. However, within these reactions, disulfides are neither created nor destroyed; rather, they are transferred from one set of thiols to another. PDI is the direct donor of disulfide bonds to folding proteins[3]. After the transfer, two endoplasmic reticulum membrane proteins, Ero1p and Erv2p, reoxidize PDI[4-6]. Ero1p and Erv2p are apparently capable of actually generating disulfides *de novo*.

Ero1p was discovered by screening for dithiothreitol-sensitive mutants and temperature-sensitive mutants defective in disulfide bond formation[7,8]. Ero1p is an FAD-dependent protein that is able to directly interact with and oxidize PDI[4,6]. Homologs of Ero1p are found in all known eukaryotic genomes, indicating that this pathway of disulfide formation is very well conserved throughout evolution.

Erv2p was isolated as a small FAD-binding protein (20 kDa) whose overexpression is capable of rescuing Ero1p mutants[5]. Erv2p appears to be part of another oxidation pathway that is able to oxidize PDI. The reaction catalyzed by Erv2p and its homologues generates disulfides directly from oxygen *via* the reaction $O_2 + RSH \rightarrow RSSR + H_2O_2$.

A new class of FAD-binding proteins

The high resolution structure of Erv2p reported in this issue by Gross *et al.*[1] reveals that Erv2p is highly α-helical, unusual for FAD-containing proteins, most of which are composed of mixed α-helices and β-sheets. In addition, the size of the FAD-binding domain and the type of electron acceptors differ from other types of FAD-binding proteins, suggesting that Erv2p represents a new class of flavoproteins. Based on sequence homology, this class appears to be widespread. Known homologs include the sulfhydryl oxidase Erv1p, a human hepatotrophic growth factor called ALR and the long studied egg-white sulfhydryl oxidase, SOX. All seem to catalyze the reaction $O_2 + RSH \rightarrow RSSR + H_2O_2$. Curiously, several members of the Erv2p family including SOX contain an additional domain homologous to PDI suggesting that these enzymes may have combined the function of Erv and PDI.

All the residues strictly conserved among the Erv2p family are located in the FAD binding site. Near this site is a Cys-X-X-Cys motif whose cysteines are connected by a disulfide. The fact that these cysteines are conserved among the family and that they are close to the catalytic site suggest their involvement in the Erv2p mechanism.

Erv2p has another pair of disulfide bonded cysteine residues present in a C-terminal Cys-Gly-Cys motif. The formation of a disulfide between two cysteine residues separated by only one amino acid is expected to be very strained, making this disulfide highly oxidizing and capable of rapid transfer to other thiol pairs.

A magician caught in the act

In an amazing stroke of luck, crystallography froze the C-terminal arm containing this second pair of cysteines in two conformations. In one conformation, the Cys-Gly-Cys motif is positioned very close to the Cys-X-X-Cys disulfide that is adjacent to the FAD cofactor. In the second conformation, the Cys-Gly-Cys motif has swung out a dramatic 10 Å away from the Cys-X-X-Cys active site. Although the exact pathway that disulfides take through Erv2p is not yet clear, one can imagine a model where disulfides generated in an FAD-dependent reaction by the Cys-X-X-Cys motif are quickly shuttled over to make a bond across the Cys-Gly-Cys motif. It is as if the magician's hands were caught in two positions, one slipping into

his hat to pluck the disulfide from its mysterious FAD source, and another where he holds the disulfide out in the open (Fig. 1). This flexible arm could be ideal for plucking the disulfide from the Cys-X-X-Cys motif and then probing into other proteins to deliver its disulfide load.

When Erv2p is overexpressed it can deliver its disulfide to PDI, but Gerber *et al*. also report that Erv2p can directly oxidize substrate proteins such as lysozyme[9]. The flexibility of the C-terminal arm containing the Cys-Gly-Cys motif may facilitate disulfide exchange between Erv2p and a variety of proteins in addition to PDI. Although essential for the function of Erv2p, the Cys-Gly-Cys motif is actually missing from Erv2p homologs — a curious observation, because residues vital for the catalytic function of enzymes are almost always well-conserved. This motif is apparently replaced by a C-terminal Cys-X-X-Cys motif in some homologs and an N-terminal Cys-X-X-Cys motif in others. In these enzymes the disulfides are apparently shuttled off to other hands that Erv2p doesn't have. The apparent ability of various parts of the Erv fold to grab the disulfide from the Cys-X-X-Cys active site provides a further indication of the importance of protein flexibility to the Erv family's catalytic mechanism.

Different strokes for proks

A similar mechanism of disulfide shuffling between two motifs has been proposed for DsbB and DsbD, two prokaryotic proteins involved in disulfide oxidation and isomerization, respectively[10-11]. DsbB is present in the inner membrane and is responsible for reoxidation of the periplasmic protein DsbA, which in turn oxidizes folding proteins[12,13]. Like Erv2p, DsbB has two pairs of disulfides. In the proposed model for DsbB, one pair reacts with DsbA and is then reoxidized by the other one[10]. This model, like that proposed for Erv2p, awaits biochemical verification.

Both the eukaryotic and prokaryotic systems utilize thioredoxin-like proteins to directly donate disulfides to secreted proteins: DsbA and related proteins in the case of prokaryotes, PDI and related proteins in the case of eukaryotes. Although there may be some similarities between the mechanisms of DsbB, the prokaryotic enzyme that generates disulfides, and of Ero1p and Erv2p, the eukaryotic disulfide generators, these enzymes display no detectable homology to each other and appear to operate by different mechanisms. Erv2p is a FAD-containing sulfhydryl oxidase that uses oxygen directly to generate disulfides via the reaction $O_2 + RSH \rightarrow RSSR + H_2O_2$ (ref. 5). Ero1p also contains FAD as a cofactor, but its oxidase activity is linked to FAD reduction rather than direct oxygen utilization[6]. DsbB does not utilize FAD at all. Rather, DsbB is a ubiquinone-containing quinone reductase[14] that donates electrons to members of the respiratory chain[15]. Under aerobic conditions, DsbB transfers electrons to ubiquinone, which then donates the electrons to the terminal cytochrome oxidases, which ultimately reduce oxygen. Unlike the Erv family, DsbB can also function under anaerobic conditions[15], where it transfers electrons to menaquinone, which then donates the electrons to alternate electron acceptors such as fumarate.

The question of where disulfides actually come from is thus starting to be answered nearly 40 years after Anfinsen first asked it. The crystallographic image of the sulfhydryl oxidase Erv2p at work is a very important advance toward solving this longstanding mystery.

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