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From structure to redox: the diverse functional roles of disulfides and implications in disease

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Abstract

This review provides a comprehensive overview of the functional roles of disulfide bonds and their relevance to human disease. The critical roles of disulfide bonds in protein structure stabilization and redox regulation of protein activity are addressed. Disulfide bonds are essential to the structural stability of many proteins within the secretory pathway and can exist as intramolecular or inter-domain disulfides. The proper formation of these bonds often relies on folding chaperones and oxidases such as members of the protein disulfide isomerase (PDI) family. Many of the PDI family members catalyze disulfide-bond formation, reduction and isomerization through redox-active disulfides and perturbed PDI activity is characteristic of carcinomas and neurodegenerative diseases. In addition to catalytic function in oxidoreductases, redox-active disulfides are also found on a diverse array of cellular proteins and act to regulate protein activity and localization in response to oxidative changes in the local environment. These redox-active disulfides are either dynamic intramolecular protein disulfides or mixed disulfides with small-molecule thiols generating glutathionylation and cysteinylation adducts. The oxidation and reduction of redox-active disulfides are mediated by cellular reactive oxygen species and activity of reductases, such as glutaredoxin and thioredoxin. Dysregulation of cellular redox conditions and resulting changes in mixed disulfide formation are directly linked to diseases such as cardiovascular disease and Parkinson's disease.

1 Introduction

Disulfide bonds have classically been shown to stabilize proteins by maintaining overall structure via intermolecular and intra-domain covalent bonds between two cysteine residues [1]. These structural disulfide bonds are essential for the stability of secreted and plasma-membrane proteins destined for the harsh oxidizing extracellular environment. Disulfide-bond formation on secreted proteins is tightly regulated by oxidoreductases in the endoplasmic reticulum (ER), including members of the protein disulfide isomerase (PDI) family [1–3]. Recent studies have shown that structural disulfide-bond formation may occur in other cellular compartments such as the mitochondria, due to the presence of oxidoreductase machineries (Erv1/Mia40) similar to the PDIs [4]. Dysregulated structural disulfide-bond formation is a hallmark of diseases such as Creutzfeldt-Jakob disease (CJD), amyotrophic lateral sclerosis (ALS) and various cancers [5, 6].

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The functional roles of disulfide bonds extend beyond the well-characterized role in protein structure stabilization (Fig. 1). Importantly, redox-active disulfide bonds regulate protein activity and localization in response to cellular redox potentials and comprise intramolecular protein disulfides and mixed disulfides with small-molecule thiols such as glutathione. Unlike static structural disulfides, redox-active disulfides are highly dynamic and are regulated by oxidative or reductive changes in the local environment [7]. Dysregulation of cellular redox potentials and the resulting aberrant formation or reduction of redox-active disulfides contributes to diseases such as thrombosis and Parkinson's [8–11]. In addition, dynamic disulfide switches are essential mediators of viral entry as demonstrated by HIV-1 [12].

This review will focus on the diverse functional roles of disulfide bonds in eukaryotic cells. The mechanisms for disulfide-bond formation in various organelles and the roles of structural and redox-active disulfide bonds in protein stabilization and regulation of activity and localization will be described. Special emphasis will be placed on the contributions of dysregulated disulfide-bond formation to human disease. Lastly, current proteomic methodologies for globally identifying structural and redox-active disulfide bonds will be surveyed.

2 Evolution of Disulfide Bonds

Cysteine residues on proteins play a variety of functional roles including metal binding, nucleophilic and redox catalysis, protein regulation and disulfide-bond formation [13]. Given the unique ability of cysteine to fulfil these diverse functional roles, it is unsurprising that cysteine is the second most conserved amino acid during protein evolution, surpassed only by tryptophan [14]. Furthermore, although cysteine is a late evolutionary addition to the genetic code, cysteine residues on proteins have accrued with higher frequency, alluding to the preferential incorporation of cysteine residues at functional loci [15]. Importantly, within a given organism, approximately 50% of cysteines are involved in disulfide-bond formation and these disulfide bonds are highly conserved. In fact, cysteines that form disulfide bonds are the most highly conserved of the amino acids (even more conserved than tryptophan), whereby 96.9% of disulfide-bonded cysteines are conserved once acquired in *H. sapiens* [16]. Interestingly, up to 99% of disulfide cysteine pairs are mutated in concert and it is rare for only one cysteine to be replaced by evolution [16, 17]. Lastly, as organismal complexity increases, there is an increased rate of accrual for disulfide-bonded cysteine residues [16], which likely reflects the need for more intricate mechanisms by which to modulate protein structure and function. In summary, the high conservation and increased rate of accrual of disulfide bonds underscores the fundamental importance of the acquisition and maintenance of disulfide bonds to eukaryotic evolution.

3 Properties of a Disulfide Bond

Protein disulfide bonds are covalent linkages between the thiol groups of two cysteine residues, which are formed via a two-electron oxidation that is often coupled to the reduction of oxygen, flavin cofactors, oxidized glutathione, and other disulfides. Disulfide-bond formation between two thiolate anions (RS^-) may occur spontaneously *in vitro* with

molecular oxygen as the final electron acceptor [1]. In contrast, within biological systems, disulfide bonds are predominately formed via thiol-disulfide exchange reactions [1], whereby a thiol nucleophile attacks an existing disulfide bond (RSSR) to yield a new mixed disulfide and free thiol species. For example, due to the high (mM) concentrations of oxidized and reduced forms of glutathione (GSSG/GSH) in the cell, the GSSG/GSH couple frequently control the formation and reduction of cellular disulfides through thiol-disulfide exchange reactions. Furthermore, oxidoreductases in eukaryotic cells including protein disulfide isomerases [18] and thioredoxin [9], accelerate the rate of disulfide-bond formation and reduction.

The redox potential of a disulfide bond, which specifies the tendency to be reduced, dictates the formation and stability of that species in a given redox environment [19]. Typically, for cellular disulfides, redox potentials are measured relative to glutathione as a standard; therefore, disulfide bonds with negative redox potentials are considered more stable and less likely to undergo thiol-disulfide exchange in the presence of glutathione. The redox potentials that have been measured for protein disulfides range from −95 to −470 mV, varying by at least 375 mV [7]. These variations in redox potential are attributed to several factors including pK_a, entropy and bond-strain energy, as discussed below [9, 19–21].

3.1 Thiol pK_a

The kinetics of thiol-disulfide exchange are dependent on the pK_a of both the nucleophilic-cysteine thiol and the leaving-group thiol. The standard pK_a for a thiol is 8.5, resulting in protonation at physiological pH. However, cysteine pK_a values are known to range from 3.5 to greater than 12 depending on the local protein microenvironment [3]. Decreased cysteine pK_a is attributed to stabilization of the thiolate by electron withdrawing groups or nearby positive charges and results in an increase in the rate of thiol-disulfide exchange [22, 23]. Low thiol pK_a values that are significantly below the pH of the solution, however, render decreased nucleophilicity of the thiolate. Therefore, the rate constant peaks when the thiol pK_a is equivalent to the pH of the solution [23]. In contrast, decreased pK_a of the leaving-group thiol increases the rate of thiol-disulfide exchange, regardless of the pH of the solution. Thiol addition to an asymmetrical disulfide (RSSR′), results in the thiol with the lowest pK_a acting as the leaving group [24].

3.2 Entropy

Entropic barriers, including rotation and translation, affect the stability of disulfide bonds and the rate of formation. Intermolecular disulfide bonds are entropically disfavored, but the entropic costs of intramolecular disulfide bonds are more complex. In denatured proteins, with large distances between cysteines, the entropic barriers to disulfide-bond formation are high due to the rotational and translational freedom characteristic of a denatured protein. As two cysteines become closer in the primary sequence, the amount of conformational freedom decreases and orientations that permit disulfide formation increases, reducing the entropic costs of disulfide-bond formation [25].

The rigid tertiary structure of a folded protein can potentially bring cysteine residues into close proximity, resulting in more thermodynamically favorable and faster disulfide-bond

formation, relative to a denatured protein. For example, a disulfide bond in bovine pancreatic trypsin inhibitor (BPTI) is stabilized by tertiary structure and therefore has been measured to display an exceptionally low redox potential of −470 mV [26].

3.3 Strain

Disulfide bonds may be unstable if the disulfide introduces strain into the protein structure. The ideal dihedral angle for disulfides is ± 90°, and studies show that distortion away from this preferred angle can increase redox potential by as much as 10–100 mV [27]. While observed in nature [28, 29], vicinal disulfides between two neighboring cysteines, and disulfides in loops, are associated with higher levels of structural strain than cysteines within more distal and less rigid regions of the protein. Furthermore, vicinal disulfide bonds reverse the direction of the peptide chain, and distort the trans-planar conformation of the peptide backbone to a cis conformation, which introduces further strain on the protein structure [30].

4 Structural Disulfide Bonds

Disulfide bonds confer structural stability to proteins destined for the secretory pathway. Importantly, secreted and plasma-membrane proteins are exposed to highly oxidizing environments (~−140 mV more than intracellular organelles) [31]. This highly oxidizing extracellular environment induces spontaneous inter- and intramolecular disulfide-bond formation [2] that disrupts protein structure. The controlled formation of structural disulfide bonds within the ER and other cellular compartments prior to secretion minimizes potential structural damage to proteins due to spontaneous extracellular disulfide formation.

Structural disulfide bonds exist as intramolecular or inter-domain disulfide bonds, which are generated in a highly controlled fashion by families of dedicated oxidoreductases [32]. Disulfide-forming oxidoreductases are abundant and essential components of the endoplasmic reticulum and include members of the PDI family [3, 33]. Similar oxidoreductase systems, comprised of MIA40/Erv1, have also been identified in the intermembrane space (IMS) of the mitochondria [4]. Furthermore, recent studies have identified mechanisms for controlled disulfide-bond formation in the most reducing organelle of the cell, the cytoplasm [34], where viruses co-opt an oxidative folding pathway for protein biogenesis [35]. The efficient and controlled formation of structural disulfide bonds is essential to maintaining protein integrity, and dysregulation of structural disulfides contributes to various types of diseases including CJD, ALS, and many cancers [5, 6, 36].

4.1 Formation of structural disulfides

4.1.1 Disulfide-bond formation in the ER; the protein disulfide isomerase family—Proteins destined for the secretory pathway are subjected to posttranslational modification (e.g. glycosylation), folding and disulfide-bond formation in the ER. The redox potential of the ER is between −150 to −180 mV with a GSH to GSSG ratio of 1:1 to 3:1, thereby rendering the ER one of the most oxidizing organelles within the cell [37]. The PDI protein family catalyzes the controlled formation and rearrangement of disulfide bonds for structural stabilization of nascent proteins. Each of the 22 PDI members contain at least one thioredoxin-like (Trx-like) domain with a thioredoxin fold [6]. These Trx-like domains may

be catalytically active (a-domain), possessing a CXXC motif, or inactive (b-domain). PDIs also possess an acidic C-terminal extension (c-domain) that terminates with an ER retention sequence [6, 38]. The PDI family members vary in the number of a and b domains that they contain [39]. For example, PDIA1 (commonly referred to as PDI), is the most abundant (~ 0.8% of all cellular protein [40]) PDI, and possesses four Trx-like domains (a, b, b′, and a′) and a c-domain with a KDEL ER retention sequence [41, 42].

In PDIA1, the redox state of the CGHC active-site motifs in the a-domains dictate if oxidase (disulfide form of PDIA1) or isomerase (dithiol form of PDIA1) chemistry is performed [43]. The non-catalytic b′ domain is responsible for identifying unfolded and improperly folded proteins through exposed hydrophobic patches on the nascent protein [44]. Oxidation of nascent proteins is accompanied by the concomitant reduction of the CGHC active-site motifs of PDIA1 [45]. Subsequent re-oxidation of the PDIA1 active sites is carried out by ER oxidoreductin 1 (Ero1), a flavin adenine nucleotide (FAD) binding oxidase [46]. Ero1 contains two cysteine pairs; one located on a flexible loop and the other two as part of a CXXC motif proximal to the FAD cofactor [47]. Oxidation of PDIA1 is first achieved through thiol-disulfide exchange with the flexible-loop cysteines, followed by transfer of electrons to the FAD cofactor via the CXXC cysteines. Lastly, these electrons are shuttled from FADH2 to O_2, producing H_2O_2 and regenerating the FAD cofactor (Fig. 2) [47, 48]. In contrast to PDIA1 oxidase activity, substrate isomerization does not result in a net change in the PDIA1 redox state, therefore, Ero1 is not required for continued isomerase activity. Isomerase chemistry occurs via the formation of a mixed-disulfide through nucleophilic attack of PDIA1 active-site cysteines on the substrate disulfide bond. The N-terminal cysteine in each CGHC active-site motif is stabilized by the adjacent histidine residue, thereby reducing the thiol pK_a (~4.5 – 5.6) [49, 50] and enabling this cysteine to act as a nucleophile in isomerase reactions. In comparison, the C-terminal cysteine has an observed pK_a of 12.8 [51]. A thiolate on the substrate subsequently resolves the mixed disulfide to produce a new disulfide whilst restoring PDIA1 to its reduced state [52]. Lastly, it is proposed that PDIA1 can also reduce disulfide bonds on substrates via formation of a mixed disulfide that is resolved by the C-terminal cysteine within the CGHC motif [53] [32]. This reductase activity may prove important for removing an incorrect disulfide when isomerization is not feasible.

In addition to PDIA1, other members of the PDI family have been shown to display oxidase and isomerase activity, including: PDIA2, PDIA3 (ERp57), PDIA4 (ERp72) and PDIA6 (ERp5) [54]. The functional redundancy of each PDI member *in vivo* has not been fully determined. It has been shown that PDIA4 expression is increased in cells in which PDIA3 levels are reduced, suggesting some functional overlap between these two PDIs [55]. Additionally, PDIA3 interacts with calnexin and calreticulin and is thought to aid in the folding of glycoproteins [54]. Future studies into the substrate specificities of the PDI members are likely to establish the unique and overlapping functions of this protein family.

4.1.2 Disulfide-bond formation in the mitochondria; Mia40—Since mitochondrial proteins are translated in the reducing environment of the cytoplasm and the mitochondria itself is a reducing environment (−280 to −340 mV), the presence of structural disulfides is surprising [56]. However, numerous mitochondrial proteins, including the Tim proteins that

comprise the translocase of the inner membrane (TIM) complex [57, 58], and the copper chaperone Cox17 [59], are known to contain structural disulfide bonds. Structural disulfides are introduced during translocation of proteins through the inner mitochondrial space (IMS), which is the most oxidizing (~−255 mV) of the mitochondrial compartments [60]. Disulfide-bond formation in the IMS uses a disulfide-relay system, Mia40 and Erv1, which is similar to the PDI/Ero1 system in the ER [61]. Mia40 (Tim40) contains a unique catalytic CPC-motif and two twin CX_9C segments that form structural disulfides with one another [62]. The catalytic CPC-motif has a redox potential of −200 mV [63], and in contrast to PDIA1, the C-terminal cysteine of the CPC-motif is the more nucleophilic of the catalytic cysteines [63]. Mia40 is responsible for disulfide-bond formation on several Tim proteins and Cox17 [4], and similar to PDIA1, re-oxidation of Mia40 is required upon disulfide-bond formation on a substrate protein. Erv1, a Quiescin-sulfhydryl oxidase (QSOX), is an electron acceptor for reduced Mia40 [64]. Erv1 contains two CXXC motifs; one located on a flexible-tail segment while the second is proximal to the FAD cofactor [64]. Electrons are transferred from Mia40 to FAD on Erv1 via the CXXC motifs, with oxidation of FADH2 occurring through transfer of electrons to two equivalents of Cytochrome c [65, 66].

The Mia40/Erv1 system in the mitochondria share similarities with the ER PDI/Ero1 system. However, several differences exist between the two systems; notably Mia40 does not contain Trx-like domains and furthermore, Erv1 belongs to a protein family (QSOX) divergent from Ero1. The substrate repertoire of Mia40 in the IMS is not fully established and it is currently unknown whether or not other disulfide-relay systems exist in the IMS.

4.1.3 Disulfide-bond formation in the cytoplasm; viral proteins—Similar to the mitochondria, the cytoplasm is considered to be highly reducing, with a redox potential of −200 to −260 mV [67], and cytoplasmic disulfide-bond formation is unanticipated. However, disulfide-bond formation occurs in keratinization as well as in in viral-protein biogenesis and has been recently reviewed [34]. Vaccinia virus was the first identified to introduce disulfide bonds on viral protein substrates in the cytoplasm [35]. A viral protein complex comprising a redox-active protein, A2.5L, and sulfhydryl oxidase, E10R, together with an accessory protein, G4L, are responsible for disulfide-bond formation [34, 35]. This disulfide-bond forming machinery is shared by several other nucleocytoplasmic large DNA viruses [34]. Additionally, the herpes simplex virus 1 (HSV-1) chaperone UL32 contains three CXXC motifs and is thought to regulate disulfide-bond formation on capsid proteins to ensure structural integrity [68]. Absence of UL32 resulted in the formation of non-native disulfide bonds, likely due to spontaneous and uncontrolled disulfide-bond formation during infection and capsid maturation [68–70]. While disulfide-bond formation in the cytoplasm is rare and only demonstrated for non-host viral proteins, it serves to exemplify that even reducing compartments of the cell are capable of hosting oxidoreductases for controlled disulfide formation.

4.2 Dysregulation of structural disulfide-bond formation in disease

Transmissible spongiform encephalopathies (TSEs): TSEs are rare and fatal disorders characterized by neurodegeneration and classified into three forms: infectious (kuru), sporadic (Creutzfeldt-Jakob disease (CJD)) and familial (fatal familial insomnia). These

disorders are the result of prion protein, PrP^{C}, misfolding into a protease-resistant form, PrP^{SC}, which is prone to aggregation (Fig. 3A) [71, 72]. PrP^{C} contains a globular domain composed of three α-helices, with a disulfide linkage connecting two of these helices [73, 74]. Upon conversion of PrP^{C} to PrP^{SC}, these α-helices are replaced by β-sheets [75]. The disulfide bridge has been shown to contribute to stabilization of the native and non-pathogenic PrP^{C} structure. When the disulfide bond on the mouse prion protein was reduced and capped, circular dichroism (CD) studies showed that the resulting reduced PrP possessed decreased α-helical content, increased β-content and larger exposed hydrophobic regions relative to its oxidized counterpart [76]. These data suggest that the disulfide bond is important to stabilize the α-helical structure of PrP and prevents exposure of hydrophobic regions that promote aggregation.

Upon translation in the ER, approximately 10% of PrP^{C} is misfolded and removed by ER-associated degradation (ERAD) (Fig. 3A) [77, 78]. An increase in misfolded PrP in TSEs causes ER stress and activation of the unfolded protein response (UPR) [78–80]. UPR activation increases expression of a variety of pro-survival genes including the PDIs [81]. For example, PDIA3 is upregulated in CJD brain tissue and protects cells from the toxicity of infectious PrP [82, 83]. PDIA3-deficient murine embryonic fibroblasts (MEFs) show increased PrP aggregation, which was further augmented by treatment with a reducing agent, suggesting that reduced disulfide-bond content correlates with increased aggregation. Furthermore, PDIA3 was shown to directly interact with PrP via co-immunoprecipitation studies [84], suggesting that either the chaperone or oxidoreductase activity of PDIA3 is important to prevent PrP aggregation.

Amyotrophic lateral sclerosis (ALS): Familial ALS (FALS) is caused by mutations in superoxide dismutase 1 (SOD1) resulting in the misfolding and aggregation of SOD1 into cross-β-amyloid fibrils that ultimately lead to cell death (Fig. 3B) [85, 86]. The first step in cross-β-amyloid fibril formation is referred to as initiation. The “nucleus” that is formed during initiation may fragment and produce fibril seeds that convert soluble SOD1 to insoluble β-rich structures and thus elongate the fibril. The process following initiation is termed seeded amyloid growth [87, 88]. Fragmented fibrils may be transmitted to new cells where they seed further aggregation [89, 90]. SOD1 exists as a homodimer with each monomer containing a β-barrel, binuclear Cu/Zn site, and an intramolecular disulfide bond [91]. The mature form of SOD1 is highly stable and therefore does not initiate aggregation [92]. Recently, fibrillation-induced fluorescence measurements identified the structural-disulfide bond in wild-type (WT) SOD1 as an important contributor to fibril formation [88]. The reduced form of SOD1 showed increased rates of seeded amyloid growth and spontaneous fibrillation, whereas no fibril initiation was observed for oxidized SOD1. Importantly, the disulfide bond in mutant SOD1 was more easily reduced than WT SOD1 and small quantities of the reduced form of mutant SOD1 were sufficient to initiate the fibrillation of WT SOD1. During seeded growth, reduced WT SOD1 was recruited faster by growing fibrils than oxidized WT SOD1, which is attributed to the conformational flexibility of the disulfide-reduced state [88]. These data identify the disulfide bond of SOD1 as an important contributor to the aggregation kinetics of both WT and mutant SOD1 in ALS.

Cancer: The rapid proliferation of cancer cells requires increased rates of protein synthesis and folding, and increased expression of folding chaperones, including members of the PDI family, is characteristic of ovarian, breast, prostate, lung and other cancers [6, 93]. Decreased PDIA1 levels in patients with breast cancer or glioblastoma has been associated with higher survival rates [94–96], and increased PDIA1 levels are associated with chemo-resistance [97]. These results together suggest that increased disulfide-bond formation and isomerization capability is essential to the survival of cancer cells. In addition to PDIA1, PDIA4 and PDIA6 levels are increased 11.2-fold and 7.75-fold, respectively, in cisplatin-resistant lung adenocarcinoma. Knockdown of PDIA4 and PDIA6 reinstated cisplatin sensitivity via a mitochondrial apoptosis or non-canonical cell-death pathway [98].

4.3 Global methods to identify structural disulfide bonds

Several methods exist to identify structural disulfide bonds in proteins through conformational changes in the protein following reduction. These methods include NMR, CD, and X-ray crystallography, and while extremely useful for identifying structural changes in a purified homogenous protein, they are not applicable for the global identification of structural disulfides within a proteome. A mass spectrometry (MS)-based method was recently developed to map native disulfide bonds on a global scale [99]. In this method, proteins are precipitated with trichloacetic acid (TCA) to maintain the integrity of disulfide linkages, and the precipitated proteins are dissolved in a denaturing buffer and free thiols capped with N-ethylmaleimide (NEM). Proteins were then digested with a combination of proteases such as Lys-C, trypsin, Glu-C, and/or Asp-N and analyzed by LC-MS/MS. Data analysis was performed using an adapted form of the p-Link software, pLink-SS, for identifying cross-linked peptides [100]. Analysis of the secreted proteome of human umbilical vein endothelial cells (HUVECs), identified more than 550 disulfide bonds.

5. Redox-active Disulfides

Redox-active disulfide bonds are reversible and responsive to changes in the redox potential of the surrounding environment, and the formation or reduction of these disulfide bonds serve to regulate protein activity and cellular localization. These disulfides can be intramolecular (oxidoreductases, allosteric disulfides, etc.) or mixed disulfides between a cysteine residue and a small-molecule thiol resulting in glutathionylated and cysteinylated adducts. Dysregulation of redox-active disulfide formation contributes to various diseases including Parkinson’s and HIV infection.

5.1. Reactive oxygen species and disulfide-bond formation

Endogenous reactive oxygen species (ROS), such as superoxide (O_2^-), the hydroxyl radical ($^{\cdot}OH$) and hydrogen peroxide (H_2O_2), are produced during cellular respiration in the electron transport chain of the mitochondria, peroxisomal activity and oxidase activity as seen during the reoxidation of PDIA1 [101–103]. Additionally, exogenous factors such as smoke, xenobiotics, radiation and other sources may generate ROS that diffuse into the cell or induce endogenous ROS production [104–106]. ROS are a common byproduct during cellular processes and play roles in cell signaling and homeostasis, however, high and persistent levels of ROS can cause oxidative damage and lead to a variety of disease states

[107]. Exposure of a cysteine thiol to ROS results in the formation of a sulfenic acid (-RSOH), which could react with GSH (glutathionylation) or a nearby cysteine to form an –inter or –intra molecular disulfide bond [108, 109]. Additionally, higher cellular GSSG levels resulting from increased ROS exposure can trigger disulfide-bond formation through thiol-disulfide exchange. ROS-induced reversible disulfide-bond formation contributes to the regulation of cellular antioxidant defense, chaperone systems and metabolism [107, 110] [111–113]. For example, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) catalyzes the conversion of glyceraldehyde 3-phophate (G3P) to 1,3-bisphosphoglycerate (1,3-BPG) in glycolysis [114]. Under oxidative stress, the active site cysteine of GAPDH is sulfenylated and subsequently forms a disulfide with a nearby cysteine [115], which limits flux through glycolysis and reroutes metabolic intermediates to the pentose phosphate pathway to generate NADPH [116]. NADPH is essential for the reduction and reactivation of thioredoxin (Trx) and glutaredoxin (Grx) [117], which are antioxidants that act to reduce cellular levels of redox-active disulfides.

5.1.1 Redox-active disulfides with small-molecule thiols

Glutathionylation: Glutathionylation refers to the formation of mixed disulfide between a protein thiol and GSSG via thiol-disulfide exchange [10, 118], or reaction of S-nitrosated or sulfenylated proteins with GSH [119]. Removal of glutathione adducts can occur spontaneously by thiol-disulfide exchange with GSH, and enzymatically by Grx, Trx or sulfiredoxin [120–122]. Various cellular pathways are regulated by glutathionylation, including the NF-κβ pathway that triggers inflammation [123]. Glutathionylation of Cys62 in the p50 subunit of NF-κβ inhibits DNA binding and downstream transcriptional activity [124]. In addition to affecting the function of transcription factors, glutathionylation can act to either inhibit or activate a variety of enzymes. Glutathionylation of protein tyrosine phosphatases (PTPs), specifically the active-site cysteine of PTP1B, inhibits phosphatase activity [125] [126]. In contrast, glutathionylation of Cys346 in cystathionine β-synthase (CBS) enhances the homocysteine to cysteine enzymatic conversion [127].

Cysteinylation: Cysteinylation involves the formation of a mixed disulfide between a protein cysteine thiol and the thiol group of free cysteine. Intracellular cysteine concentrations have been found to be around 100 μM, approximately a magnitude lower than glutathione concentrations [128]. Cysteinylation is analogous to glutathionylation; although, it remains poorly characterized in eukaryotic systems [129]. Cysteinylation has been observed in human serum albumin [130] and a recent study showed an increase in cysteinylation of plasma protein thiols with age [131]. Additionally, SOD1, which catalyzes the dismutation of superoxide anions into molecular oxygen and hydrogen peroxide [132], was found to be cysteinylated in human neuronal tissue [133]. *In vitro* studies showed that cysteinylated SOD1 is protected from oxidative damage [133]. The small number of characterized human cysteinylated proteins is likely due to the scarcity of methods to study this modification, and as technologies for enrichment and identification improve, the targets of cysteinylation may prove to be more prevalent.

5.1.2. Redox-active disulfides on oxidoreductases

Thioredoxin (Trx): The Trx family is composed of two Trx systems, Trx1 (12 kDa) and Trx2 (18 kDa), that catalyze thiol-disulfide exchange [117, 134, 135]. Each Trx is composed of a thioredoxin-fold (four α-helices and five β-sheets) and a CGPC (CXXC) active-site motif [134]. Trx1 is found extracellularly [136] and intracellularly in the cytoplasm and nucleus while Trx2 is localized to the mitochondria [9]. Trx1 is responsible for activation of extracellular proteins, regulation of the intracellular redox environment, and modulation of transcription factors and gene expression in the nucleus [137, 138]. Trx2 regulates the mitochondrial redox environment and protects cells from oxidant-induced apoptosis [139]. The redox potentials of the CXXC motifs of the Trx proteins are around −270 mV rendering them some of the most reducing of the oxidoreductases [140]. Similar to PDIA1, the N-terminal cysteine in the active site has a low pK_a, ~ 6.3 [141], and acts as the nucleophile for thiol-disulfide exchange reactions. The mixed disulfide is resolved by the C-terminal active-site cysteine yielding oxidized Trx and a reduced substrate [142]. Oxidized Trx is reduced by the dimeric flavoenzyme, thioredoxin reductase (TrxR) [143], which utilizes NADPH as the electron donor (Fig. 4A) [143]. Trx activity is regulated by Trx-interacting protein (Txnip), which binds to the active site and inhibits Trx1 reductase activity [144]. Overexpression of Txnip has been shown to slow cell growth [145] and stimulate apoptosis [146]. Trx activity is also regulated by glutathionylation of Cys73, a non-active site cysteine [147]. Interestingly, nitrosation of Cys73 results in Trx gaining trans-nitrosation activity, whereby the NO moiety is transferred to the active-site cysteine of caspase-3 [148]. Members of the thioredoxin system (Trx, NADPH, TrxR, and Txnip) are implicated in various aspects of (patho)physiology, including embryonic development, neurodegenerative diseases, viral infections, cardiovascular diseases, cancer and aging [9, 149].

Glutaredoxin (Grx): Grx proteins constitute a family of relatively small oxidoreductases (~9–15 kDa) [150], primarily localized in the cytosol, nucleus and mitochondria [151] (human Grx1 is also known to be secreted [152]). Grx proteins contain a Trx-like fold and either dithiol (CXXC) or monothiol (CXXS), active-site motifs for reducing protein disulfides or glutathionylation adducts [151, 153, 154]. The mechanism for protein-disulfide reduction by dithiol Grx proteins is analogous to that described for PDIA1 and Trx. The resulting oxidized dithiol Grx is re-reduced after each catalytic cycle by GSH to form reduced Grx and GSSG [151]. For monothiol Grx proteins that act on glutathionylated substrates, the resulting Grx-glutathione mixed disulfide is resolved by a second molecule of GSH to form GSSG and reduced monothiol Grx [154] [155]. The cellular GSSG:GSH ratio is restored by glutathione reductase and NADPH [117], whereby electrons are transferred from NADPH to GSSG via a redox-active disulfide on glutathione reductase to regenerate GSH (Fig. 4B) [117]. Due to the high specificity displayed by Grx proteins for reducing glutathionylated proteins, this family of proteins are important players in cellular pathways regulated by protein glutathionylation [118]. Additionally, Grx proteins can reduce intra- and inter-protein disulfide bonds on ribonucleotide reductase [156], peroxiredoxin [157], and collapsing response mediator protein 2 (CRMP2) [158], thereby affecting proliferation, detoxification and developmental pathways, respectively. For example, CRMP2 was shown to be reduced by Grx2, and silencing of Grx2 in zebrafish resulted in increased levels of oxidized CRMP2 and subsequent neuronal loss by apoptotic cell death [158]. Lastly,

dysregulated Grx activity is implicated in various diseases, including Parkinson's disease and HIV infection [159].

5.1.3 Allosteric redox-active disulfides—Allosteric redox-active disulfide bonds are typically distal to functional loci within the protein, but induce a functional change in the protein upon reduction by oxidoreductases or thiol-disulfide exchange [160, 161] [12]. Functional changes elicited by allosteric disulfides include changes in ligand binding, substrate hydrolysis, proteolysis, and oligomerization [12]. Allosteric disulfides are not defined by a common sequence motif, but do share similarities in the disulfide-bond geometry. Disulfide bonds are classified by the five chi (χ) angles of the six atoms that make up the bond, C_α-C_β-S_γ-S_γ'-C_β'-C_α', whereby each χ angle can be positive or negative, rendering twenty possible disulfide geometries. Three of the twenty configurations, -right-hand staple (−RHstaple), -left-handed hook (−LHhook) and −/+RHhook (−/+RHhook), are enriched in allosteric disulfide bonds. Of these, −RHstaples are enriched six-fold, and LHhooks and −/+RHhooks are enriched threefold in allosteric disulfides relative to all disulfides [161, 162]. −RHstaples and −/+RHhooks were found through computational studies to be more stressed than other geometries, due to stretching of the S-S bond and bending of the neighboring bond angles [163]. In contrast, −LHhooks show similar bond stress when compared to the other 17 disulfide configurations and it remains unknown as to why this configuration is enriched in allosteric disulfides. Interestingly, −RHstaples are often cross-strand disulfides that link adjacent strands in a β-sheet, and the proximity of the strands generate a highly strained disulfide bond [7, 161]. Cleavage of this strained disulfide bond can therefore induce large conformational changes. For example, reduction of the intramolecular −RHstaple between Cys130-Cys159 in CD4 results in domain swapping and the formation of an intermolecular disulfide-linked homodimer [164–166].

Allosteric disulfide-bond reduction by oxidoreductases is governed by several factors. Importantly, steric factors, including the accessibility of the disulfide and accommodation of the disulfide in the oxidoreductase active site, contribute significantly to disulfide-bond recognition and reduction by oxidoreductases. Furthermore, the environmental context, in terms of which oxidoreductases are present in the local vicinity, is also important in determining which disulfides are reduced by which oxidoreductases [11]. Additionally, disulfide-bond reduction occurs via S_N2 nucleophilic substitution, whereby the three sulfurs involved in disulfide bond reduction must be oriented in a 180° angle [167]. Stretching and twisting of proteins may change the orientation of the three sulfurs making it easier or more difficult for disulfide cleavage to occur [168]. Lastly, allosteric disulfide-bond reduction by oxidoreductases is also dictated by the redox potentials of the disulfide and the oxidoreductase active-site redox couple. The redox potential for allosteric disulfides range from −184 mV to −255 mV [12], and lie within the redox potential range for oxidoreductases (−120 mV to −270 mV) [169]. Most allosteric-disulfide redox potentials are more negative than PDIA1, thereby necessitating a stronger reductase such as thioredoxin. However, allosteric disulfide cleavage has been performed by PDIA1, PDIA6, and potentially PDIA3 [170–173]. The number of characterized allosteric disulfide bonds remains relatively small, amounting to ~30 across mammals, bacteria, plants, and viruses [162], and are implicated in the pathogenesis of various diseases and viral infection [162,

170]. Ongoing efforts to structurally define characteristic features of allosteric disulfides are likely to expand the identification and underlying functions of these motifs [12]. For a more comprehensive review on allosteric disulfides refer to references: [11, 12, 137, 162].

5.2 Contributions of redox-active disulfide bonds to disease

Oxidative stress is the result of an imbalance between ROS and antioxidant defenses leading to a deviation from the resting redox state of the cell, and is implicated in a variety of diseases including neurodegenerative diseases (Parkinson's Disease and ALS) [8], cardiovascular disease [174] and cancer [175]. High GSSG/GSH ratios and increased disulfide-bond formation are often characteristic of these diseases [176, 177]. For example, a recent study showed that a cysteinylated cysteine in human serum albumin (HSA), Cys34, may be used as a marker for oxidative stress-related diseases using blood samples of patients with chronic liver disease, chronic kidney disease, or diabetes mellitus [178]. Redox-active disulfides that are directly linked to disease pathology are described below. Future identification of similar inter- or intra-molecular disulfides could yield a wider range of biomarkers for diagnosis and therapeutic targets for treatment of diseases characterized by oxidative stress.

Glutathionylation of DJ-1 in Parkinson's disease: Parkinson's disease (PD) is a progressive neurodegenerative disease that affects the central nervous system and is characterized by the formation of Lewy bodies and the death of dopaminergic neurons in the *substantia nigra* [179]. Oxidative stress is implicated in the pathology of PD and mutations in DJ-1, an antioxidant protein, correlate with early onset PD in humans [8, 180]. Oxidizing conditions result in sulfenic acid formation or glutathionylation of Cys106 in DJ-1 [181] [182], and mutations to this residue increase sensitivity to the neurotoxin 1-methyl-4-phenylpyridinium (MPP^+). Interestingly, Grx1 knockdown is associated with a decrease in DJ-1 protein levels, suggesting that deglutathionylation of DJ-1 by Grx1 protects DJ-1 from degradation [183]. The protective function of Grx1 is further supported by the observation that Grx1 levels are significantly decreased in the dopaminergic neurons of PD patients [184]. Additionally, overexpression of DJ-1 in a *C. elegans* model of PD containing a GLRX-10 (Grx1 homolog) knockdown provided dopaminergic neuronal protection [182], confirming that glutathionylation of DJ-1 and potentially other proteins may contribute to the pathogenesis of PD.

Allosteric redox-active disulfide bonds in HIV-1 gp120 and CD4: Human immunodeficiency virus-1 (HIV-1) is a lentivirus that ultimately causes acquired immunodeficiency syndrome (AIDS). Viral entry of HIV-1 into host cells requires interactions between the HIV viral envelope protein, gp160, and host cell surface co-receptors, CD4 and chemokine receptor, CXCR4 or CCR5 [185]. The gp160 envelope protein is proteolytically cleaved into two subunits: gp120, a disulfide-rich surface glycoprotein, and gp41, a transmembrane protein, which non-covalently interact to form a trimer of gp120-gp41 heterodimers. Upon binding to CD4 and CXCR4, gp120 and gp41 dissociate and initiate fusion of the viral and host membranes [186].

Of relevance is the fact that both gp120 and CD4 contain allosteric redox-active disulfide bonds, which require reduction prior to viral entry [12]. Reduction of disulfide bonds on gp120 unmasks the fusion peptide in gp41 for membrane insertion (Fig. 5A) [170]. PDIA1 is responsible for gp120 reduction and inhibition of PDIA1 prevents membrane fusion [170, 187]. Moreover, six of the nine disulfide bonds in gp120 have been shown to be reduced by PDIA1 [170], Trx [188, 189] and/or Grx [159]. Recently, MS analysis using a Trx kinetic trapping mutant identified a disulfide bond in the V3 domain of gp120, Cys296-Cys331, as a target of Trx [189]. Importantly, this disulfide bond is in the −RHStaple configuration, which is a hallmark of many allosteric redox-active disulfides. Furthermore, binding of CD4 to gp120 enhances the reduction of Cys296-Cys331, postulated to be due to conformational changes in the V1/V2 and V3 loops of gp120 that increases interactions with oxidoreductases [190]. Lastly, reduction of the Cys296-Cys331 disulfide induces conformational changes in the V3 loop that ultimately activate the fusogenic properties of gp41 [189].

Similar to gp120, CD4 contains a disulfide bond in its second domain, Cys130-Cys159, which is categorized as a −RHStaple configuration with high strain energy, thereby fulfilling the criteria of an allosteric redox-active disulfide [12, 164]. *In vitro* reduction of this metastable disulfide was shown to induce a structural collapse in the second domain and increase thermal stability in the first two domains of CD4 [191]. This disulfide is reduced by Trx secreted by $CD4^+$ T cells [164], and promotes the formation of CD4 disulfide-linked dimers. CD4 dimerization enhances binding to the major histocompatibility complex class II (MHCII) and triggers the immune response [166]. Importantly, preventing dimer formation through cysteine mutation increases HIV entry [192] [193], alluding that the monomeric form of CD4 is preferential for efficient HIV entry (Fig. 5B). Recombinant 2-domain CD4 (2dCD4) bound to gp120 was unable to undergo Trx-mediated dimerization *in vitro* suggesting that gp120 may inhibit CD4 dimerization and prevent the formation of MHC-II antigen presenting complexes [194].

In addition to inducing structural changes, changes to the redox state of CD4 also regulates CD4 membrane localization [195]. Lateral movement of CD4 results in partitioning into different membrane microdomains, including detergent-resistant membranes (DRMs)/“lipid rafts” and detergent-soluble membranes (DSMs)/“non-rafts” [196, 197]. Importantly, the lateral movement of CD4 appears to be redox regulated, whereby inhibition of Trx-1 induces translocation of CD4 into DRMs [195]. CD4 localization in DRMs has been shown to be necessary for HIV infection [198, 199]. However, it has also been shown CD4 localization to DSMs supports HIV entry [197, 200]. Although contradictory, these studies suggest the dynamic interplay between cell-surface redox activity, modulation of allosteric disulfides on CD4, and microdomain localization of CD4, on HIV infection.

Allosteric redox-active disulfides in hemostasis and thrombosis: Hemostasis is a process that involves the coagulation of blood at the site of damaged blood vessels. Hypercoagulation can lead to hemostatic disorders such as thrombosis, in which excessive blood clotting obstructs blood flow in the circulatory system. Allosteric disulfide bonds in blood proteins such as β2-glycoprotein I [201], β3 integrin [202] and von Willebrand factor (VWF) [203] are implicated in hemostasis and thrombosis. VWF is a multimeric plasma

glycoprotein that mediates platelet adhesion at the sites of injury [204]. Low levels of VWF lead to von Willebrand's disease, which is characterized by excessive bleeding, and high levels of VWF can lead to thrombosis [205, 206]. The shear forces of flowing blood induce conformational changes in VWF from coiled balls to elongated structures that self-associate into a meshwork that binds platelets (Fig. 5C) [207]. Mutagenesis and MS studies of VWF showed that thiol-disulfide exchange at two disulfide bonds in the C2 domain of VWF results in self-association of VWF to form VWF oligomers [203]. The proposed mechanism involves oxidoreductase-mediated cleavage of the Cys2431-Cys2453 disulfide, followed by intermolecular thiol-disulfide exchange to form a VWF dimer. Similarly, oxidoreductase-mediated cleavage of a second functional disulfide in the C2 domain, Cys2451-Cys2468, promotes trimer formation. This disulfide bond is categorized as a −RH staple, congruent with allosteric disulfides. An in-depth overview of allosteric disulfides on blood proteins are provided in reference [11].

5.3 Global methods to identify structural disulfide bonds

Global methods to identify and quantify redox-sensitive disulfides are fundamental to fully illuminate the physiological roles of functional disulfides. This section will summarize recent developments in proteomic methods to identify redox-sensitive disulfides. Adaptation of standard two-dimensional electrophoresis (2DE)-based separation methods can provide facile visualization of all oxidized cysteines within a proteome. Differentiation of oxidized and reduced cysteines is achieved using the following sequence of steps; (1) cap reduced thiols with a thiol-reactive agent such as iodoacetamide (IAM) or N-ethylmaleimide (NEM); (2) reduce oxidized cysteines with reducing agents such as dithiothreitol (DTT) or tris(2-carboxyethyl)phosphine (TCEP); (3) derivatize these newly reduced cysteine thiols with radiolabeled [208] or fluorescent [209, 210] derivatives of IAM or NEM; and (4) separate proteins by 2DE and visualize by autoradiography or fluorescence [211]. Whilst 2DE provides facile visualization of oxidized proteins within a sample, the poor reproducibility and necessity to individually process each gel spot for protein identification by MS, limit the applicability of this method.

Shotgun-proteomic strategies that utilize isotope-coded affinity tags (ICAT) have been adapted for redox proteomics. ICAT reagents are biotinylated-IAM derivatives containing isotopically light or heavy linkers [212], which allow for cysteine tagging and quantitative monitoring of the relative abundance of cysteine-containing peptides using the light:heavy signal intensities. Initial applications of ICAT to redox proteomics used a similar workflow to the 2DE methods, where reduced cysteines were capped with IAM, and oxidized cysteines were reduced and capped with ICAT reagents for enrichment on streptavidin beads and subsequent identification of oxidized cysteines using MS. Light and heavy ICAT reagents were used to compare oxidized cysteines in control (heavy ICAT) to hydrogen peroxide-treated (light ICAT) proteomes [213, 214]. In a later iteration of this method, termed OxICAT, quantification of the extent of oxidation was performed by using the light and heavy ICAT reagents to cap reduced and oxidized cysteines within a single sample (Fig. 6A) [215].

Analogous to the ICAT methods, a cysteine-reactive iodoacetamide-alkyne (IAA) probe has been used to identify hundreds of reactive cysteines within a complex proteome [216]. This method, termed isoTOP-ABPP, and derivatives thereof [217], have been applied to identify oxidized, nitrosated, and electrophile-modified cysteines [218–220]. Oxidation of a dithiol to a corresponding disulfide can be detected by the characteristic loss in thiol reactivity induced by disulfide-bond formation. This loss in reactivity is reflected in reduced labeling of disulfide-bonded cysteines by the IAA probe and can be quantified by the incorporation of characteristic isotopic tags to oxidized and reduced samples for MS analysis (Fig. 6B). Prior applications of this strategy focused on IAA probe addition to cell lysates, which was non-ideal due to the artefactual introduction of disulfides and other oxidation events during the process of cell lysis. The high cytotoxicity of the IAA probe precludes treatment of living cells with high concentrations of this reagent. To overcome this high cytotoxicity, a latent, non-toxic derivative of a cysteine-reactive probe was developed. This caged-bromomethylketone (caged-BK) probe shows significantly decreased cytotoxicity relative to IAA and allows for temporal and spatial control of cysteine labeling in living cells (Fig. 6C). The caged-BK probe was applied to identify protein oxidation events, including disulfide-bond formation, triggered by growth-factor stimulation and subsequent intracellular hydrogen-peroxide release [221].

Importantly, 2DE, oxICAT and isoTOP-ABPP-based methods for redox proteomics are unable to differentiate between different forms of oxidation and selectively identify disulfides. Although methods specific for inter- and intra-molecular protein disulfides are lacking, proteomic platforms are available to selectively identify glutathionylated cysteines within a proteome (Fig. 7A). These include the use of biotinylated derivatives of glutathione, such as BioGSH, BioGSSG, or the cell permeable BioGEE [222] [223], which allow for enrichment of glutathionylated proteins on streptavidin, and subsequent release of glutathionylated peptides/proteins with reducing agents for MS identification (Fig. 7B). The added bulk of the biotin group in these glutathione derivatives could affect the protein targets of glutathione modification, and furthermore, necessitates the addition of exogenous biotinylated glutathione at non-physiological levels, which does not allow for monitoring endogenous glutathionylation events. To overcome these limitations, a recent method developed a mutant of glutathione synthetase (GS), capable of coupling γGlu-Cys to azido-Ala to form azido-GSH (Fig. 7B). The generation of a GSH-analog with a built-in bioorthogonal handle allows for enrichment and subsequent identification of glutathionylated proteins [224, 225]. Lastly, a Grx-mediated reduction method was used in place of DTT or TCEP to selectively reduce glutathionylation adducts for gel and MS analysis, allowing for targeted identification of glutathionylated residues within proteomes (Fig. 7C) [226]. Further development of sensitive and selective methods for identifying the various types of redox-active disulfides is critical for further characterizing the role of disulfides in regulating catalytic activity and protein-protein interactions.

6. Conclusion

Disulfide bonds are highly dynamic and are important contributors to both protein structure and function. Functional disulfides regulate protein folding and structural stabilization, sense and control changes in the redox environment, and regulate protein activity, localization and

interactions via disulfide breakage or formation. This diverse array of functions illustrates the importance of disulfide bonds in biological systems. Dysregulation of all aspects of protein disulfide bonds can contribute to disease pathogenesis. As such, the protein levels and redox states of oxidoreductases, the undesired reduction of structural disulfides, oxidative stress-induced disulfide formation and thiol-disulfide exchange-mediated reduction of allosteric disulfides, all have substantial roles in various diseases. Methods for identifying and characterizing functional disulfides continue to improve and may lead to the discovery of new functions for disulfide bonds and reveal uncharacterized diseases regulated by disulfide-bond formation. Pharmacological targeting of oxidoreductases, and modulation of cellular redox levels, can provide potential avenues for therapy for these wide array of diseases.

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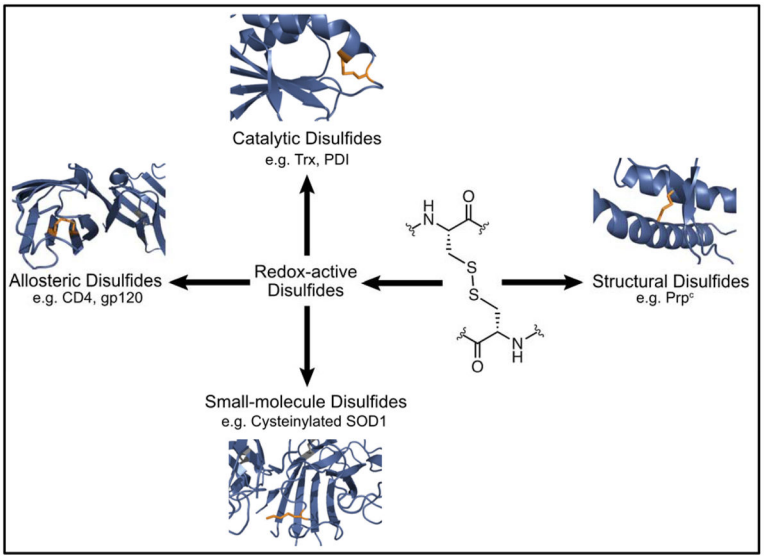

Figure 1.

Characterized functional roles of disulfides: structural and redox-active disulfides. Redox-active disulfides may be further characterized as small-molecule, catalytic and allosteric disulfides.

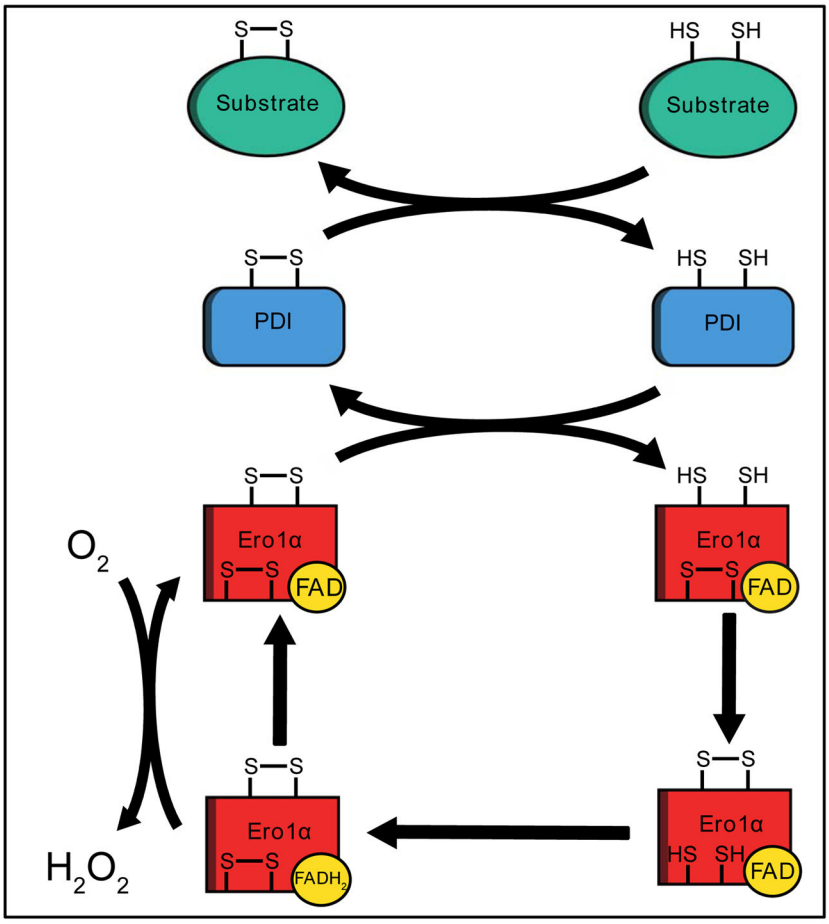

Figure 2.

The Ero1α-PDI-mediated oxidative protein-folding pathway. PDI is reoxidized by Ero1α, a FAD-binding oxidase. Ero1α transfers electrons from PDI to FAD with oxygen being the final electron acceptor.

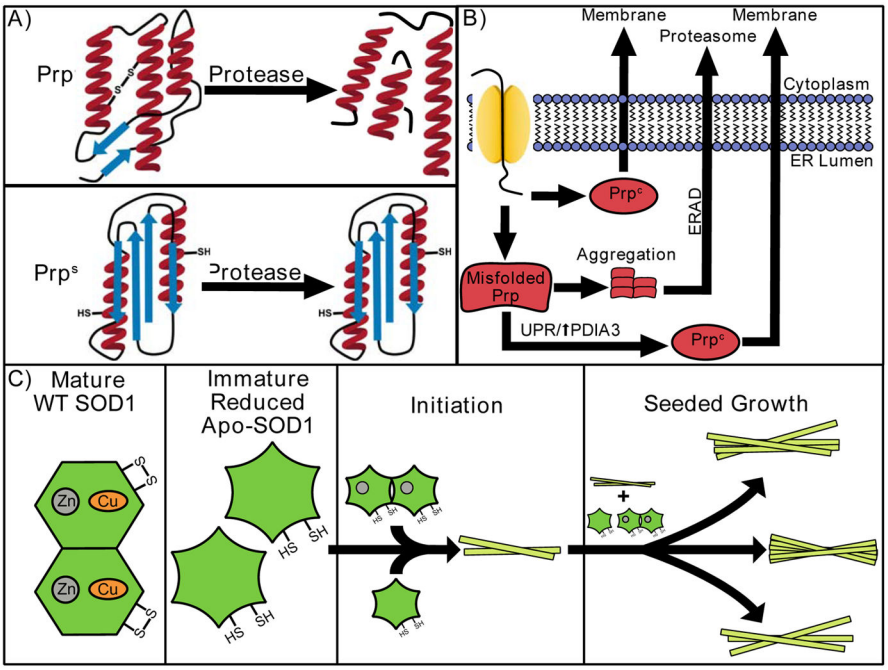

Figure 3.

Structural disulfides in disease. (A) The disulfide bond in Prp^{C} is reduced in Prp^{SC}, which is protease-resistant. (B) Misfolded Prp is removed from the ER and degraded via ERAD. Accumulation of misfolded Prp leads to activation of the UPR, resulting in increased expression of folding chaperones such as PDIA3, which interacts with misfolded Prp to re-fold and prevent aggregation. (C) Immature and disulfide-reduced apo-SOD1 or Zn-SOD1 initiates the formation of cross-β-amyloid fibrils creating a “nucleus.” The “nucleus” fragments to produce “seeds” that convert soluble SOD1 into insoluble β-rich structures, elongating the cross-β-amyloid fibrils.

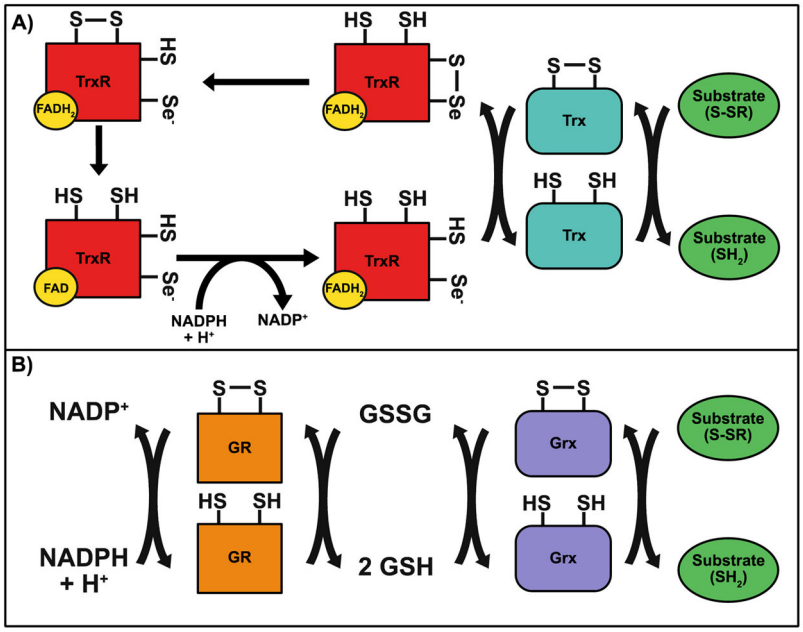

Figure 4.

The Trx-TrxR and Grx-GR-mediated disulfide-reduction pathways. (A) Upon reduction of substrate proteins, the resulting oxidized Trx is reduced by TrxR. TrxR transfers electrons from NADPH, via a bound FAD and a cysteine-cysteine and cysteine-selenocysteine couple to the oxidized Trx. (B) Similar to Trx, Grx reduces disulfide bonds in substrate proteins. Grx is reduced by GSH, yielding GSSG. GSH levels are restored by GR via the transfer of electrons from NADPH to GSSG via a redox-active disulfide on GR..

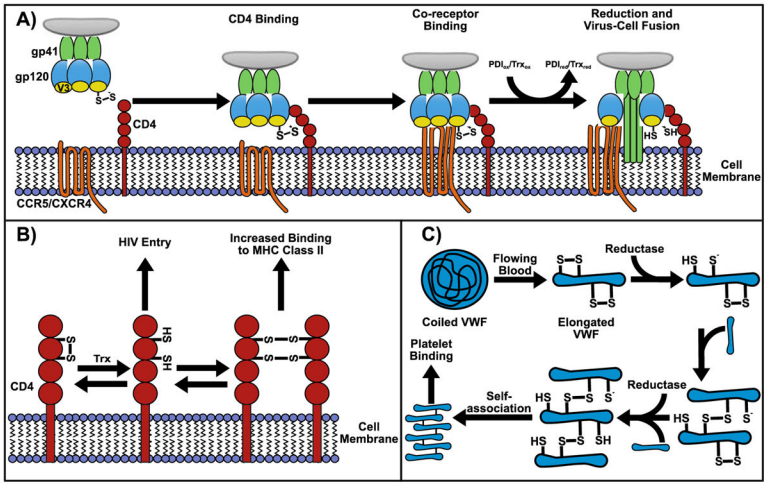

Figure 5.

Redox-active disulfides in disease. (A) Reduction of a disulfide bond in gp120 by PDI or Trx promotes HIV virus-host cell fusion by conformational changes that expose the fusion peptide on gp41 for membrane insertion. (B) Reduction of a disulfide bond on CD4 results in the formation of CD4 homodimers linked by disulfide bonds. Dimeric CD4 shows increased binding to MHC Class II. The reduced monomer of CD4 is preferred for HIV entry. (C) The shear force of flowing blood induces conformational changes in VWF from a coiled ball to an elongated structure. Intramolecular disulfides in elongated VWF may be reduced by an oxidoreductase, promoting intermolecular disulfide bond formation and the self-association of VWF, which promotes platelet binding.

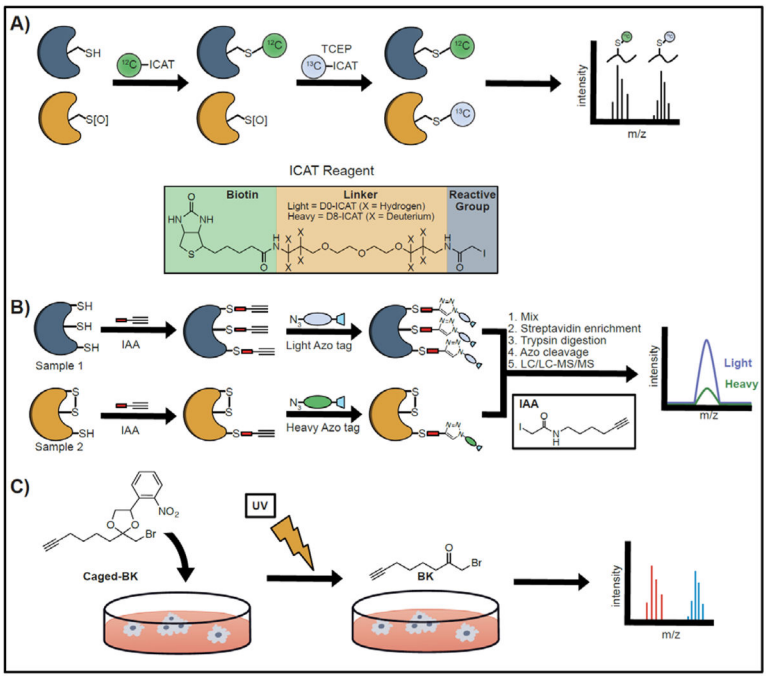

Figure 6.

Proteomic methods to study redox-active disulfides. (A) The OxICAT method incorporates light and heavy ICAT reagents (inset) to reduced and oxidized cysteines, respectively, for analysis by MS. (B) isoTOP-ABPP utilizes a cysteine-reactive iodoacetamide-alkyne (IAA) probe (inset) to identify reactive cysteines within a proteome. Cysteine oxidation to form disulfides is accompanied by a characteristic loss in cysteine reactivity that can be quantified by incorporation of light and heavy tags (Azo-tags) to IAA-modified peptides for MS analysis. (C) Caged-bromomethylketone (Caged-BK) contains a masked cysteine-reactive electrophile that can be activated *in situ* by UV irradiation.

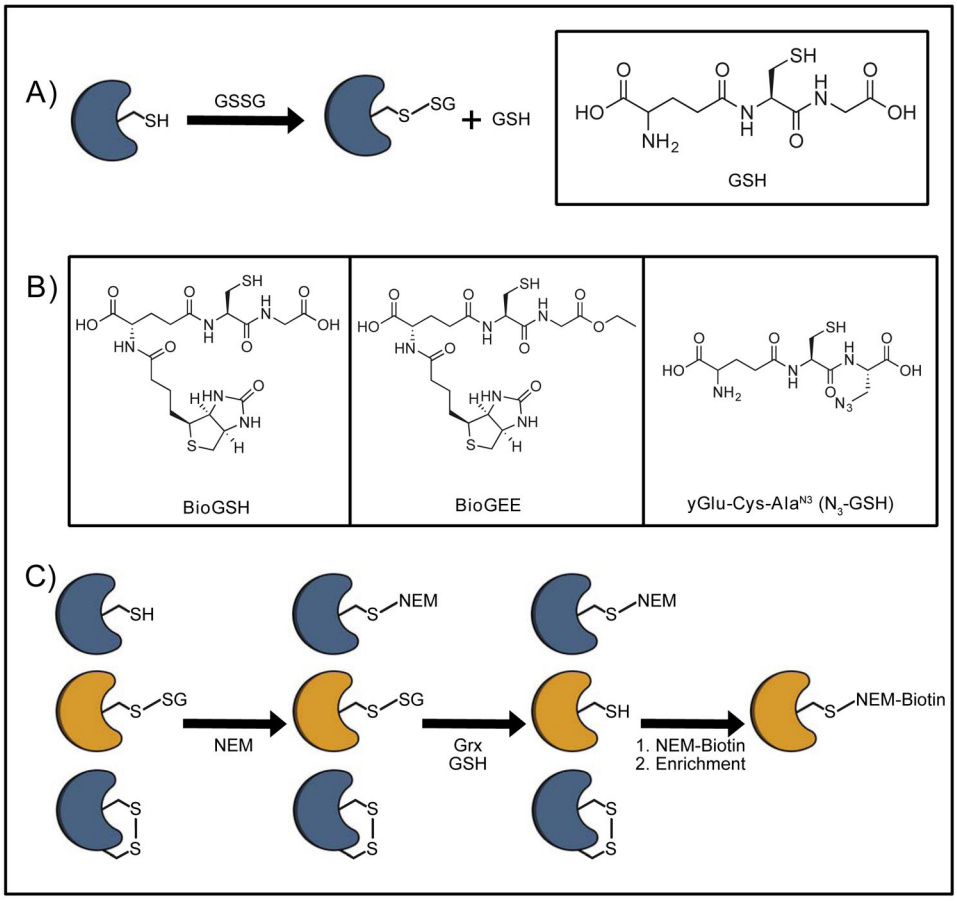

Figure 7.

Proteomic methods to study glutathionylated proteins. (A) Glutathionylation results in the formation of a mixed disulfide between a protein and glutathione. (B) Biotinylated glutathione adducts used to study glutathionylation include BioGSH, BioGEE and N_3GSH. (C) Glutathionylated proteins can be identified by capping reduced cysteines with NEM followed by Grx-mediated reduction of glutathionylated cysteines. The resulting newly-formed thiols are then capped with NEM-biotin for avidin-biotin enrichment of glutathionylated proteins.