Allosteric disulfides: Sophisticated molecular structures enabling flexible protein regulation

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Disulfide bonds are one of the first elements of protein architecture we learn about in biology. Although they seem simple at first glance, they are a key part of what made human life possible. An analysis of the time of acquisition and conservation of 5,181 protein disulfide bonds across 29 eukaryotic species remarkably showed that about three-quarters of disulfide bonds in human proteins were acquired in vertebrate ancestors (1). Moreover, the acquisition of a disulfide bond coincided with the evolution of a new protein in most cases, and there was a positive trend in the rate of disulfide acquisition and complexity of the organism. Once a disulfide bond appeared in a protein, it was very rarely lost thereafter. These findings suggest that accrual of disulfide bonds has contributed to the higher complexity of vertebrates. It is of interest that the frequency of cysteine in the proteome of modern species is increasing (2, 3), which implies that disulfide acquisition is ongoing in our evolution.

For a long time, disulfide bonds were assumed to be inert in the mature protein. However, we now know that disulfide bonds play both structural and functional roles. Structural disulfides are those that are formed during protein folding and either impact the folding process and/or stabilize the resultant protein architecture. There are two types of functional disulfide: the catalytic disulfides that manipulate disulfides in other proteins, and allosteric disulfides that control the function of the protein in which they reside when cleaved or formed (4, 5). Allosteric control of protein function is defined as a change in one site—the allosteric site—that influences another site by exploiting the protein’s flexibility (6). An allosteric disulfide bond thus represents the “allosteric site,” and the conformational change triggered by cleavage of the bond alters protein function. Although there is some ambiguity in the structural versus functional designation, it is helpful to define disulfide bonds in this way. For instance, while the “structural” disulfides are iner they can contribute to protein function by stabilizing the structural integrity of the native conformation and associated binding sites.

The first disulfide bond implicated in protein control was identified in the mid-1990s in the adhesive glycoprotein thrombospondin-1 (TSP-1). TSP-1 is secreted by mammalian cells and participates in cell–cell and cell–matrix interactions. Ligand binding to TSP-1 was shown to be controlled by protein-disulfide isomerase (PDI)2 that manipulates a disulfide bond in the C-terminal domain of the protein (7–11). About 40 allosteric disulfides have now been described (Table 1) where we know the identity of the disulfide and the consequences for protein function when the bond is cleaved. The bonds have been identified in viruses, bacteria, plants, and mammals. They have been arranged in Table 1 according to the type of change in the protein upon cleavage of the disulfide: ligand binding, substrate hydrolysis, proteolysis, or oligomerization. There are many other allosteric disulfides under investigation that have yet to be defined at this level of understanding.

This review focuses on allosteric disulfides and the factors that cleave them. The concept of allosteric disulfides was intro---
Another to achieve a net negative protein as it folds is negative, the change in entropy of the spontaneous reaction. Although the change in entropy of the system cannot decrease. Protein folding is a process where the thermodynamics of this spontaneous process are governed by Gibbs free energy.  

$\Delta G = \Delta H - T \Delta S$

This equation is dominated by the second law, $\Delta G = \Delta H - T \Delta S$, which states that the entropy of a closed system cannot decrease. Protein folding is a process where the enthalpy ($\Delta H$) and entropy ($-T \Delta S$) terms compete with one another to achieve a net negative $\Delta G$, which is required for a spontaneous reaction. Although the change in entropy of the protein as it folds is negative, the change in entropy of the surrounding water molecules is positive, i.e. reductions in conformational entropies are balanced by gains in entropy due to release of solvent molecules. The enthalpic contributions include hydrogen bonding of the protein and water molecules, ionic salt bridges, van der Waals forces and, of course, disulfide bond formation (Fig. 1). Heat is released during formation of these bonds.

In eukaryotic cells, disulfide bonds form in the ER, Golgi complex, post-Golgi complex vesicles, and mitochondrial inter-membrane space. In bacteria, these bonds form in the periplasmic space. The formation of disulfide bonds in all these compartments is assisted by many protein factors and small molecules, primarily the oxidoreductases, chaperones and GSH. The information required for the folding of a protein is encrypted within its primary amino acid sequence. The role that disulfide bonds play in folding has been debated, and different theories have been proposed. A popular concept is that disulfide bonds direct protein folding in a stepwise fashion where a protein achieves its native structure via stable disulfide intermediates that are partially folded. Another model says that disulfide bonds stabilize the native conformation rather than facilitate folding. It is based on conformational sampling of the native structure, either locally or globally, and lastly formation of disulfide bonds that stabilize the mature conformation. It has also been proposed that disulfide formation drives protein folding, i.e. the local changes induced by the formation of a single disulfide bond directs global unfolding of the protein in a cooperative fashion. It is likely that all models are in play to some extent and that their relative contributions to the native structure will vary from protein to protein.

Where disulfide bonds are found

Over 180,000 disulfide bonds are present in Protein Data Bank structures. Many disulfides are represented more than once in different structures of the same protein. For example, immunoglobulin disulfides are represented thousands of times. A subset of 13,031 unique disulfide bonds in 4,895 nonredundant human proteins is shown in Table S1 along with a number of features of the bonds. About half of these disulfide bonds are in membrane proteins (plasma or organelle membranes) and about half in proteins containing a secretion signal sequence (secreted or function in the ER, Golgi, and endosome). Interestingly, 298 proteins that function in the cytoplasm and/or nucleus contain 509 structurally-defined disulfide bonds. These disulfide bonds can be grouped in Table S1 by sorting on the “Subcellular location” column. This group is notable as the cytoplasm and nucleus are cellular compartments traditionally not thought to support disulfide bond formation because they are considered too reducing. How many of these disulfides form in the proteins in their native environment remains to be determined, although it seems likely that several of them will. For instance, we know that disulfide bonds form in some prokaryote and eukaryote transcription factors. We also know that different intracellular environments have different redox potentials that will influence disulfide bond stability. Formation or cleavage of a protein disulfide bond is a lot about the protein being in the right place at the right time.

### Table 1

Examples of allosteric disulfides grouped according to the type of change in protein function associated with cleavage of the bonds

| Change | Protein | Disulfide Cys | Reference |
|--------|---------|--------------|-----------|
| Binding | B2-glycoprotein 1 | 288-326 | (70) |
|          | B3 integrin | 177-184 | (51) |
|          | HIV gp120 | 126-196, 296-331, 385-418 | (77/82) |
|          | CD4 | 130-159 | (64) |
|          | CD44 | 81-101 | (83) |
|          | Glycoprotein 1b | 4-17 | (84) |
|          | Interleukin-4 | 46-99 | (70) |
|          | Interleukin receptor subunit gamma | 160-209 | (85) |
|          | Thrombospondin-1 | 997-97 | (7,11) |
|          | Tissue Factor | 186-209 | (69,86) |
|          | Vitronectin | 137-161 | (87) |
|          | von Willebrand factor | 274-453 | (69) |

**Disulfide Hydrolysis**

| Protein | Disulfide Cys | Reference |
|---------|--------------|-----------|
| Adenovirus 2-phospholipid kinase | 86-119 | (88) |
| Arylsulphate sulfotransferase | 418-424 | (89) |
| Botulinum neurotoxins | 436-445 | (90) |
| QueF | 55-99 | (91) |
| Thiols | 88-378 | (92) |
| Trypsinase | 191-220 | (93) |
| Methionine aminopeptidase 2 | 228-448 | (94) |
| Prolyl cis-trans isomerase, AIFKB13 | 106-111 | (95) |
| Transglutaminase 2 | 370-371 | (42) |
| Lom protease | 230-370 | (96) |
| CD2Dm | 83-221 | (97) |
| SAMHD1 | 341-350 | (98) |
| Argininosuccinase | 18-138 | (99) |
| Factor XI | 362-482 | (100) |
| MKC | 292-259 | (101) |
| Plasminogen | 512-536 | (57) |
| Protease | CD4 | 130-159 | (64) |
| Vascular endothelial growth factors C & D | 156-165 | (58) |
| LYVE-1 | 201-201 | (183) |
How allosteric disulfides are cleaved

Protein disulfide bonds can be cleaved by oxidoreductases and by thiol–disulfide exchange (Fig. 2). Another mechanism, recently discovered in an engineered system, is hydrolysis of the disulfide. All three mechanisms of disulfide bond cleavage are potentially reversible.

Oxidoreductases

Humans make more than 20 different oxidoreductases (26, 27), and many of them are exported by activated cells. Seven members of the PDI family (26), two members of the thioredoxin family, and glutaredoxin-1 (27) are secreted into the blood (28), whereas transmembrane TMX1, TMX3, ERP44, and ERp29 have been detected on the platelet surface (29–31). An important fundamental question is how the secretion of oxidoreductases is achieved and regulated. They are not exported by the classical secretion pathway.

The active sites of oxidoreductases contain a reactive dithiol/disulfide that reduces or oxidizes a substrate disulfide bond (Fig. 2A). For example, an active-site dithiol will reduce a substrate disulfide bond and become oxidized in the process. For the oxidized oxidoreductase to cleave another substrate disulfide, it needs to be first reduced in another reaction. Oxidoreductases are not catalysts in the true sense of the word as the bonds are consumed during reaction and require regeneration. The suffix ‘ase’ is used to designate an enzyme, although the oxidoreductases may be more correctly considered factors rather than enzymes. A conundrum in the field is how or if these factors turn over in the extracellular space. There are mechanisms in the ER and cytoplasm that regenerate spent oxidoreductases. For instance, cytoplasmic thioredoxin is reduced by cytoplasmic thioredoxin reductase using electrons from NADPH (27). Such reactions have not been identified.
outside the cell. A mechanism of oxidation of an oxidoreductase has been described—oxidation of PDI by platelet surface Erp1α (30)—but no mechanism of reduction of the factors has been found thus far. It is possible that some oxidoreductases act as single turnover reductants in the extracellular space (32), i.e., a reduced oxidoreductase is secreted and cleaves one substrate disulfide bond. Although this scenario seems a waste of energy in protein production and secretion, it may be an energy cost that cells will tolerate. The flip side is that the activity of single turnover enzymes is readily controlled—they act once and are then cleared. This situation avoids the need for endogenous inhibitors of the factors, which have not been identified for secreted oxidoreductases to date.

Secreted oxidoreductases appear to have many substrates in the extracellular milieu (33). The following general parameters likely govern substrate specificity of the oxidoreductases. As for proteases and their substrates, lock-and-key-type interactions of the substrates with the binding pockets of the factors will be important, particularly considering the stereochemistry of disulfide bond cleavage. Reduction of a disulfide bond proceeds via an S_n2 reaction mechanism where the sulfur ion nucleophile of the oxidoreductase and the two sulfur atoms of the substrate disulfide bond must line up at an ~180° angle for cleavage to proceed (34, 35). In fact, distorting a protein influences the rate of disulfide bond cleavage by changing the alignment of the sulfur atoms (36, 37). Other factors that will influence substrate specificity are spatial (right place) and temporal (right time) considerations and matching the energetics of the oxidoreductase and the substrate disulfides. An oxidoreductase that exhibits a lower redox potential (more negative value) will only donate electrons to or cleave the disulfide of a substrate with a higher redox potential (more positive value).

Transglutaminase-2 (TG2) provides one recent example where location and redox potentials are critical elements in defining disulfide bond regulation. TG2 is ubiquitously expressed in mammals and has a wide range of functions. Inside the cell, TG2 functions as a GTPase and participates in cell signaling. Outside the cell, TG2 converts glutamine residues on substrate proteins to glutamate. The enzyme is also an autoantigen in celiac disease (38). Enzyme assays coupled with structural studies have defined an allosteric disulfide linking cysteines 370 and 371 as a switch that turns TG2 on (reduced) or off (oxidized) in the extracellular matrix (Fig. 3A). Ex vivo and in vivo studies have shown that the bond is reduced by thioredoxin (39, 40) and oxidized by Erp57 (41) in the extracellular space. The protein conformational transitions triggered by cleavage or formation of the bond mediate allosteric control of enzyme activity (42). The Cys-370–Cys-371 disulfide has a standard redox potential of −184 mV (39), which fits with the redox potentials of the switching oxidoreductases. Thioredoxin has a redox potential of −270 mV (43), which is in accordance with its function as a TG2 reductant, whereas the redox potentials of the two catalytic dithiols/disulfides of Erp57 are −167 mV (α-domain) and −156 mV (α′-domain) (44), which is consistent with its role as TG2 oxidizer.

Vascular thiol isomerases—The role of oxidoreductases and their substrates in thrombosis and hemostasis is a particularly active area of research. This activity has been stimulated by findings that the extracellular actions of five oxidoreductases, the vascular thiol isomerases PDI (45), Erp57 (46), Erp5 (47), Erp72 (48), and TMX1 (31), are essential for normal thrombosis in mice (31, 49). PDI, Erp5, Erp57, and Erp72 are soluble proteins secreted by activated platelets and endothelial cells, whereas TMX1 is an intrinsic membrane protein of the platelet plasma membrane. Interestingly, the soluble vascular thiol isomerases are pro-thrombotic in vivo, whereas TM1 is anti-thrombotic. Platelet-specific knockouts of the individual soluble vascular thiol isomerases all result in decreased platelet incorporation into growing thrombi, whereas knockout of TMX1 increases platelet incorporation.

A function of one of the vascular thiol isomerases, Erp5, was recently elucidated. Blood vessel damage leads to blood platelet aggregation at the injury site. The aggregation is largely mediated by cross-linking of platelet αllbβ3 integrin receptors on different platelets by the bivalent circulating ligand, fibrinogen. It is critical that platelet aggregation is spatially constrained so that the platelet plug does not occlude the blood vessel and damage the tissue supplied by the vessel. One mechanism by which this process appears to be controlled is through secretion of Erp5 from activated platelets (47, 50). Erp5 binds to the β3 subunit of activated αllbβ3 integrin and cleaves the β-domain Cys-177–Cys-184 disulfide bond at the rim of the fibrinogen-binding pocket (51). Cleavage of the disulfide changes the conformation of the binding pocket and triggers release of fibrinogen (Fig. 3B).

It is not clear at present whether the five vascular thiol isomerases work independently or cooperatively to control thrombosis. It has been proposed that they may function as a redox chain (49), shuffling electrons among themselves and to substrate disulfides. The PDI family factors have been shown to exchange electrons with each other (52), so this scenario is feasible. Examination of the redox potentials of the soluble vascular thiol isomerases provides, in principle, support for this idea (Table 2). It is conceivable that electrons flow from thioredoxin to Erp72 to Erp5 to PDI to Erp57, and to substrate disulfides at any point in the chain (Fig. 4). It is also possible that electrons could skip Erp5 and flow directly from Erp72 to PDI, for example. Outstanding questions for this model are the original source of the electrons and whether there are other as yet unknown factors in the chain.

Pathological thrombosis is the first human disease where allosteric disulfides are being targeted for treatment. The flavonoid quercetins were discovered in a high-throughput screen of an annotated library of known bioactive compounds to be inhibitors of the vascular thiol isomerase, PDI. Quercetin–3-rutinoside blocks PDI activity by binding to the substrate-binding pocket and inducing a conformational change that impairs protein substrate binding (53). Isoquercetin, which has a glycoside at the third position on the C ring, has improved bioavailability in humans and was selected for clinical development (54). It is membrane-impermeable, orally-available, inhibits PDI activity and thrombus formation in mice and plasma PDI activity and platelet-dependent thrombin generation in humans. Isoquercetin is currently being tested as an anti-thrombotic in a cancer thrombosis clinical trial (54).
Evidence for involvement of oxidoreductases in human disease, whether detrimental or protective, has mainly been correlative thus far. The first molecular evidence for a causative role came from exome sequencing that identified rare SNPs in oxidoreductases associated with amyotrophic lateral sclerosis (ALS) (55). A total of 16 novel SNPs in PDI and ERp57 were identified in patients with familial or sporadic ALS that are not present or are rarely found in healthy controls.

Of the SNPs, four variants (PDID292N, PDIR300H, ERp57D271N, and ERp57Q481K) have been studied with respect to effect on PDI and ERp57 function in protein maturation in the ER and a role in pathogenesis of ALS (56). Expression of the mutant oxidoreductases in murine motoneuron cell lines impairs dendritic branching and thus neuronal outgrowth. The functional consequences of three of the mutations have been studied. The PDI D292N mutation alters PDI interaction with its ER substrates by restricting substrate access to the binding pocket, whereas the R300H mutation has the opposite effect. The ERp57 D271N mutation creates a new N-linked

**Table 2**

| Oxidoreductase | Domain     | Disulfide  | $E^\prime_0$ | Refs. |
|---------------|------------|------------|--------------|-------|
| Thioredoxin   |            | Cys-32–Cys-35 | −270         | 43    |
| ERp72         | a          | Cys-91–Cys-94 | −217         |       |
|               | a’         | Cys-206–Cys-209 | −215       |       |
|               | a”         | Cys-555–Cys-558 | −220     |       |
| ERp5          | a          | Cys-55–Cys-58 | −206         | 51    |
|               | a’         | Cys-190–Cys-193 | −211     |       |
| PDI           | a          | Cys-53–Cys-56 | −191         | 104   |
|               | a’         | Cys-397–Cys-400 | −190    |       |
| ERp57         | a          | Cys-57–Cys-60 | −167         | 44    |
|               | a’         | Cys-406–Cys-409 | −156     |       |

*J. Chiu and P. Hogg, unpublished data.

**Oxidoreductase SNPs and disease**—Evidence for involvement of oxidoreductases in human disease, whether detrimental or protective, has mainly been correlative thus far. The first molecular evidence for a causative role came from exome sequencing that identified rare SNPs in oxidoreductases associated with amyotrophic lateral sclerosis (ALS) (55). A total of 16 novel SNPs in PDI and ERp57 were identified in patients with familial or sporadic ALS that are not present or are rarely found in healthy controls.

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**Figure 3. Allosteric disulfides in transglutaminase-2 and αIIbβ3 integrin.** A. Cys-370–Cys-371 disulfide is a switch that turns TG2 “on” (reduced) or “off” (oxidized) in the extracellular matrix. The bond is reduced by thioredoxin and oxidized by ERp57. Control of enzyme activity is via conformational transitions in the protein upon cleavage or formation of the disulfide. The disulfide cysteines are in yellow, and local conformational changes are highlighted in blue ribbon. The oxidized TG2 structure is of PDB identifier 2q3z (42) and reduced structure of 1kv3 (105). The surface representation of the thioredoxin and ERp57 structures are of PDB identifiers 1aiu (106) and 3f8u (107), respectively. B, ERp5 (red oval) triggers fibrinogen release from activated platelet αIIbβ3 integrin by cleaving the β3-domain (cyan oval) Cys-177–Cys-184 disulfide bond. Molecular dynamics simulations show that cleavage of the disulfide results in long-range allosteric effects within the β3-domain, including in the metal-binding sites that are critical for fibrinogen binding. The blue sticks represent the allosteric signaling network measured by force distribution analysis (51). The network involves both cysteines, Asp-119 that is critical for ligand binding, and Asp-217 and Asp-174 that are involved in positioning of the calcium (yellow spheres) and magnesium (orange sphere) ions. The αIIb β-propeller domain is the green cartoon and the β3-domain the cyan cartoon.
glycosylation site in the protein that impacts ERp57 function in ER homeostasis. It is not known whether these SNPs impair function of the secreted oxidoreductases and their cleavage of allosteric disulfides, although it is likely that they will.

Thiol–disulfide exchange

Cleavage of disulfides by thiol–disulfide exchange occurs within or between proteins or between proteins and small molecules. In contrast to oxidoreductases, this cleavage does not require additional electrons (Fig. 2B). For intramolecular reactions, all that is required is a conformational change in the substrate protein to bring the three sulfur atoms involved in-line (i.e. the sulfur ion nucleophile and the two sulfur atoms of the disulfide bond). This mechanism of cleavage could be triggered by ligand binding and mechanical forces, for instance. Allosteric disulfide bonds in plasminogen (57) and vascular endothelial growth factors C and D (58), for example, are cleaved by thiol–disulfide exchange.

Hydrolysis

A hydrolytic mechanism of cleavage of a disulfide bond (Fig. 2C) was recently discovered in an engineered protein (59). A tetrameric metalloprotein with disulfide bonds at the subunit interfaces was constructed, and removal of the tetramer’s four Zn$^{2+}$ ions resulted in cleavage of one of the bonds. A sulfenic acid/thiol pair was resolved in the X-ray structure of the Zn$^{2+}$-depleted protein, which implies hydrolytic cleavage. This finding is the first example of this mechanism of cleavage, and its significance for native disulfide bonds remains to be determined.

**Why allosteric disulfides are cleaved**

As mentioned above, the reduction of a disulfide bond depends on geometry of the atoms involved and the energetics of the disulfide bond to be cleaved. A protein’s backbone and the geometry of the disulfide bonds that link it can change shape (60). For instance, the Cys-119–Cys-205 disulfide bond in the HIV envelope glycoprotein, gp120, can adopt five different conformations in different structures of the protein (60). However, the current indications are that allosteric bonds strongly favor one conformation only. To appreciate the significance this finding, we must first discuss how disulfide conformation is classified and how conformation influences bond cleavage.

Our laboratory devised a classification system for disulfide bonds when we first described allosteric disulfides in 2006 (4). We thought at the time that allosteric bonds might prefer certain conformations, and we needed a scheme to classify disulfides based on conformation. The knowledge gained since 2006 supports this hypothesis. The classification system is founded on the geometry of the five dihedral or $\chi$ angles that describe the cystine residue (4). The dihedral angle in chemistry is the angle between planes through two sets of three atoms, having two central three dihedral angles define whether the bonds are spiral ($-,-,-$ or $+,-,-$), hooks ($+,+,-$ or $+,+,+$), or staples ($-,+,-$ or $-,+,+$. The S–S bond dihedral angle determines whether the conformation is right-handed ($+$) or left handed ($-$). The signs of the two $\alpha$–$\beta$ dihedral angles determine...
Figure 5. Classification of disulfide bonds and allosteric conformations. A, classification of disulfide bonds using the five dihedral angles of the cystine residue (center). There are 20 possible disulfide conformations using these angles, and example structures of each from the PDB are shown. B, incidence of the three allosteric disulfide conformations. The −RHstaple, −LHhook, and −/+RHhook conformations constitute −20% of all nonredundant disulfide bonds in the PDB, but −80% of the structurally defined allosteric bonds (n = 32 (61)). C, allosteric −RHstaple and −/+RHhook conformations are naturally strained. The pairwise forces between the cysteine residues of the 20 different disulfide conformations were calculated using force distribution analysis (65). The stresses on the −RHstaple and −/+RHhook disulfides are significantly higher than the other 18 conformations. The bonded pre-stress (or tensile pre-stress) measure comprises all force terms involving the sulfur atoms of the disulfide, so is the relevant indicator for disulfide reactivity. Mean tensile pre-stress of the 20 disulfide conformations was adapted from Fig. 2D of Zhou et al. (65). D, tensile pre-stress of the −RHstaple and −/+RHhook bonds is a result of stretching of the sulfur–sulfur bond length (indicated by d) and neighboring α angles.

whether the bond is termed − (both angles are negative), + (both angles are positive), −/+, or +/−. Twenty possible disulfide bond conformations are possible using this convention, and all 20 are represented in protein structures (Fig. 5A).

The −LHspiral conformation accounts for about one-quarter of all disulfide bonds and is often associated with stable, typically structural bonds (4). The disulfides at the active sites of oxidoreductases are nearly always +/−RHhooks because they occur in the same relatively rigid thioredoxin-domain fold. Allosteric disulfide bonds are most often associated with one of three conformations (Fig. 5B). The −RHstaple is the archetypal allosteric conformation and accounts for 14 of the 36 structurally characterized allosteric disulfides to date (61, 62). There are eight examples of −LHhooks and seven examples of −/+RHhooks, so far. The protein secondary structures linked by the −RHstaple bonds have some recurring features. These disulfides often link adjacent strands in antiparallel β-sheets, also known as cross-strand bonds (63, 64), adjacent (vicinal) cysteines in the polypeptide chain and constrain β-loop structures. The secondary structures linked by −LHhooks and −/+RHhook bonds, however, are more variable, and no pattern is apparent at this time. For example, the TG2 Cys-370–Cys-371 disulfide links adjacent cysteines and has a −RHstaple conformation (42), whereas the allBβ3 integrin Cys-177–Cys-184 bond links a hydrogen-bonded turn and a loop structure and has a −/+RHhook conformation (Fig. 3) (51). The reasons why the −RHstaple and −/+RHhook conformations are associated with allosteric function are beginning to emerge.

In essence, the protein’s structure prevents the −RHstaple and −/+RHhook disulfides from fully relaxing into the state of minimal potential energy, so the bonds carry a “tensile pre-stress” (Fig. 5C) (4, 65). The stress mostly derives from stretching of the S–S bond and neighboring α angles (Fig. 5D) and is expected to fine-tune disulfide cleavage and thus the function of the protein. In support of this scenario, stretching of S–S bonds has been demonstrated experimentally to increase their susceptibility to cleavage (35–37, 66). The natural strain of the −RHstaple and −/+RHhook disulfides may have contributed to their evolution as allosteric bonds. Their propensity for cleavage coupled with a positive functional change would have resulted in evolutionary pressure to retain the bonds. In contrast, the −LHhook conformation is no more stressed than the
other 18 conformations. It is an open question why this bond type associated with allosteric function. It is possible the −LHhook conformation is better suited to one type of cleavage, such as thiol/disulfide exchange (Fig. 2B), but this hypothesis remains to be examined. Interestingly, structural and molecular dynamics studies of the tissue factor (67, 68), CD4 (64, 65), von Willebrand factor (69), and integrin β3 (51) allosteric disulfides show that they always adopt the same conformation. The tissue factor, CD4, and von Willebrand factor disulfides always have a −RHstaple conformation, whereas the integrin β3 allosteric bond is always a −/+/RHhook. This situation is due to topological features that constrain the secondary protein structure in which the bond resides. Such constraints likely set the bond up for cleavage and also enable it to reform when required. In contrast, dynamic isomerization of certain allosteric disulfide bonds is possibly an important component of their function, i.e. cleavage of the bond may only occur when the disulfide adopts a particular conformation in response to events such as ligand binding or mechanical forces. For instance, the Cys-46–Cys-99 disulfide in interleukin-4 that is cleaved by extracellular thioredoxin to inactivate the cytokine (70) can adopt 10 different conformations in NMR structures of the soluble protein, including an allosteric −/+/RHhook conformation. This particular scenario has yet to be tested experimentally.

Tools and techniques for exploring allosteric disulfides

As mentioned above, TSP-1 was the first protein discovered to have an allosteric disulfide. The key ingredient to this discovery was the identification of a mAb that recognizes different disulfide-bonded forms of TSP-1. The antibody was used to show that cultured mammalian cells make the different TSP-1 forms and that PDI changes the balance of the forms. Of course, techniques have advanced since the 1990s, but the identification and study of functional disulfide bonds are being held back by technical hurdles. There are some simple tools that point to the presence of a redox-active disulfide in a protein, such as reporter-tagged thiol-reactive compounds that react with the cysteine thiols of reduced allosteric bonds. Biotin-linked maleimides are often used and protein labeling measured using streptavidin-based probes. Change in incorporation of the label is an indication of cleavage or formation of an allosteric bond. A shortcoming of this technique is that it does not provide a quantitative measure of disulfide cleavage or formation in the protein population. Does the event occur in 0.1 or 50% of the protein molecules? If it is occurring in only a minor fraction of the protein, it may not be relevant. Also, if the protein being studied has more than one disulfide bond, which is often the case, this technique does not indicate which is being cleaved.

To properly analyze an allosteric disulfide, it is important to quantify its redox state in the protein population and how this state shifts under different conditions relevant to the protein in question. Unfortunately, this measurement is far from routine at present. The best method currently is a differential cysteine alkylation and MS technique (51, 71). Reduced disulfide bond cysteines in the protein are first alkylated with thiol-specific 2-iodo-N-phenylacetamide ([12C]IPA). The protein is then resolved on SDS-PAGE, the disulfide bonds reduced with DTT, and the disulfide cysteines alkylated with a carbon-13 isotope of IPA ([13C]IPA). The protein is digested, the peptides separated using LC and their identity determined by MS. The LC peak areas of peptides containing the disulfide bond cysteines labeled with either [12C]IPA or [13C]IPA are calculated. The ratio of [13C]IPA to [12C]IPA + [13C]IPA for the peptides represents the fraction of the disulfide in the protein population that is in the reduced state. The advantage of this pair of cysteine alkylators is that they have the same efficiency of reactivity and the same structure, which enhances the reliability of alkylation and resolution of the alkylated peptides by LC and their detection by MS. A mass difference of 6 Da is the only change in a cysteine-containing peptide labeled with [13C]IPA or [13C]IPA. This method was used to measure the redox state of 24 of the 28 disulfide bonds in the β3 subunit of platelet αIIbβ3 integrin (51). The downside is that the method takes several months to perfect for a new user and needs to be optimized for each protein.

What the field needs is a method to quantify disulfide bond cleavage that is as easy and quick to use as the routine method to quantify peptide bond cleavage. The study of proteolytic control of protein function languished until the advent of SDS-PAGE that enabled precise quantification of this chemical event. The equivalent of an SDS-PAGE-type method to quantify allosteric disulfide bonds in complex protein mixtures would enable rapid advances in our understanding of and appreciation for this post-translational modification.

How to target allosteric disulfides

As most allosteric disulfides function outside the cell, they are excellent drug targets. Inhibitors do not need to cross the plasma membrane to function, which also markedly reduces the chances of off-target effects. Allosteric disulfides can be targeted by both biologicals and small molecules. Monoclonal antibodies could take out either the reduced or oxidized form of a protein or the factor that cleaves or forms the bond. Small molecules can be designed to cleave an allosteric disulfide or prevent one from forming. They can also be designed to inhibit the cleaving factors, such as the PDI inhibitor isouquercetin (see above).

Cleavage of an allosteric disulfide bond can be achieved using targeted cysteines. For example, an allosteric disulfide bond in the HIV envelope glycoprotein, gp120, has been targeted using this approach (72, 73). HIV gp120 binds to CD4 and a chemokine receptor on immune cells that leads to infection of the cell. CD4 mimetics that contain a cysteine sulfur ion nucleophile bind to gp120 and cleave the Cys-126–Cys-196 allosteric disulfide, which results in a stable mixed disulfide linking the two molecules and inhibition of HIV infection.

The reduced form of an allosteric disulfide can be targeted using thiol-reactive moieties that will prevent the bond from re-forming (74). Co-location of an allosteric protein cysteine thiol with a poorly reactive thiol alkylator will result in specific alkylation of the allosteric cysteine, rendering it unable to reform the disulfide. Thiol alkylators containing weak electrophilic moieties such as chloroacetyl, acrylamide, or aminomethyl methyl acrylate are useful for this purpose. These compounds react very slowly with unpaired cysteines unless they
contain a targeting moiety that brings them into close proximity with the allosteric cysteine. For instance, specific covalent coupling of a ligand containing a chloroacetyl group with a cysteine in an antibody-binding pocket was achieved in mouse ascites fluid (110).

The future of allosteric disulfides

Allosteric disulfides are proving to be an important aspect of protein function across biological systems and life forms. It is only relatively recently that investigators have become aware of these bonds and are finding more and more each year. Considering that about 1 in 5 disulfides in proteins in the Protein Data Bank have 1 of the 3 allosteric conformations (Fig. 5B), there may be hundreds, if not thousands, of allosteric disulfides. Indeed, it may be that as many disulfide bonds are cleaved in proteins as peptide bonds. As a cleaved disulfide bond can reform, as seen in the example of TG-2, allosteric disulfides have the potential to reform, unlike peptide bonds. This reversibility provides for a more sophisticated control of protein function compared with proteolysis and is perhaps one of the reasons why disulfide bonds have accrued faster in complex organisms (1).

It is a helpful starting point to consider that a potential allosteric disulfide exists in an equilibrium between cleaved and bound forms in the protein population and that perturbation of the equilibrium state one way or the other has different functional consequences. When studying an allosteric disulfide, it is therefore wise to consider factors that will cleave or form the bond and not presuppose which event is more relevant. However, some cleaved disulfides will not reform due to downstream effects, such as proteolysis of the protein. This situation is the case for the allosteric disulfides in plasmin (57).

A surprise is that disulfide bonds are not limited to secreted proteins, which was traditionally thought to be their purview. About 300 proteins that function in the cytoplasm and/or nucleus contain about 500 structurally-defined disulfide bonds (Table S1) (61), and some of these bonds appear to have an allosteric role. How these bonds form in the cytoplasm and nucleus and how their redox state is controlled in these environments are important questions. PDI, the archetypal oxidoreductase that was originally thought to reside only in ER, has been found associated with the actin cytoskeleton (75). This observation suggests that PDI may facilitate disulfide bond formation in the cytosol, as it does in the ER.

Allosteric disulfides are a supermarket of new drug targets. The most advanced drug in this class is isoquercetin, the PDI inhibitor that is in Phase II testing in a cancer thrombosis trial (54). This trial is an important milestone in the field and is the first of what will likely be many attempts to target this post-translational modification for the treatment of disease. We anticipate that the next 2 decades will see therapeutics that target allosteric disulfides being approved for treatment of unwanted thrombosis and other human conditions.

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