Allosteric disulphide bonds as reversible mechano-sensitive switches that control protein functions in the vasculature

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Abstract

Disulphide bonds are covalent linkages of two cysteine residues (R-S-S-R') in proteins. Unlike peptide bonds, disulphide bonds are reversible in nature allowing cleaved bonds to reform. Disulphide bonds are important structural elements that stabilise protein conformation. They can be of catalytic function found in enzymes that facilitate redox reactions in the cleavage/formation of disulphide bonds in their substrates. Emerging evidence also indicates that disulphide bonds can be of regulatory function which alter protein activity when they are cleaved or formed. This class of regulatory disulphide bonds is known as allosteric disulphide bonds. Allosteric disulphide bonds are mechano-sensitive, and stretching or twisting the sulphur-sulphur bond by mechanical force can make it easier or harder to be cleaved. This makes allosteric disulphide bonds an ideal type of mechano-sensitive switches for regulating protein functions in the vasculature where cells are continuously subjected to fluid shear force. This review will discuss the chemistry and biophysical properties of allosteric disulphide bonds and how they emerge to be mechano-sensitive switches in regulating platelet function and clot formation.

Keywords Disulphide bonds · Protein functions · Vasculature

Introduction

Disulphide bond is a covalent linkage between two cysteines connected by the two sulphur atoms. Unlike hydrolysis of a peptide bond, cleavage of disulphide bond is reversible as it can be cleaved but also re-formed by the activity of a class of enzymes known as oxidoreductases (Sevier and Kaiser 2002). Disulphide bonds, which can connect distant domains to close proximity, are important structural elements for stable protein conformation. Structural disulphide bonds are mostly

considered as inert in the mature protein (Chiu and Hogg 2019). Disulphide bond forms catalytic motif in the active site of oxidoreductases, which facilitates oxidation and reduction reactions (redox) involved in the cleavage, formation, or isomerisation of disulphide bonds in substrate proteins (Sevier and Kaiser 2002). Many oxidoreductases such as protein disulphide isomerase (PDI), ERp5, ERp57, and ERp72 are chaperone proteins residing in the endoplasmic reticulum to shuffle disulphide bonds in a polypeptide chain for correct protein folding (Appenzeller-Herzog and Ellgaard 2008).

More recently, disulphide bonds are found to be involved in regulatory function beyond their role in protein stability and catalysis. They can act like switches to induce a change in protein functions and thus exert allosteric control on proteins. This class of disulphide bonds are known as 'allosteric disulphide bonds', where the allosteric site (disulphide cleavage or formation) triggers a functional change at another site in the protein (Chiu and Hogg 2019; Schmidt et al. 2006). Mechanical force has been shown to influence redox chemistry of disulphide bonds in vitro (Baldus and Grater 2012; Beedle et al. 2018; Giganti et al. 2018; Wiita et al. 2006) and more recently in vivo to impact the functions of proteins in blood coagulation (Butera et al. 2018; Passam et al. 2018). This review will discuss the chemical and biophysical

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properties of allosteric disulphide bonds and their role as mechano-sensitive switches to regulate protein functions in the vasculature.

Thiol and disulphide bond chemistry

Cysteine is the second most highly conserved amino acid after tryptophan in the evolution of proteins (Jordan et al. 2005). This is not surprising given the versatility of the thiol (–SH) sidechain on a cysteine, which can afford the amino acid to additional functions besides disulphide bond formation, such as metal ion coordination, thiol oxidation, S-nitrosylation, S-glutathionylation or S-palmitoylation that are important means of protein regulation in biological processes (Marino and Gladyshev 2012). In a given organism, approximately 50% of cysteines are involved in disulphide bond formation, and these bonds are highly conserved making disulphide cysteines the most conserved amino acid that surpasses tryptophan. Gain of cysteines in proteins also occurs at a higher frequency than other amino acid (Hurst et al. 2006; Jordan et al. 2005). More importantly, once acquired, disulphide bond cysteines are generally retained across the different species. In fact, 81% of disulphide cysteines never undergo mutation, but if they are mutated, both cysteines involved in a disulphide bond tend to mutate together rather than a single disulphide cysteine alone (Wong et al. 2011).

Disulphide bond cleavage or reduction is a S_N2 type nucleophilic substitution mechanism, in which one bond is broken, while another one is formed synchronously (Nagy 2013). The reaction, also known as thiol-disulphide exchange, is highly directional requiring a nucleophilic sulphur ion (thiolate anion) to attack a sulphur of the disulphide moiety only when all three sulphur atoms are aligned at 180° (Fig. 1a). Thiol-disulphide exchange can be an intra- or intermolecular reaction such that a thiolate anion can originate from a cysteine residue in vicinity to the disulphide bond in the same protein or from a cysteine in another protein such as an oxidoreductase (Fig. 1b, c). Reduction of a disulphide bond is governed by its redox potential, which specifies its stability and tendency to be reduced in a given redox environment. Redox potentials for protein disulphide bonds range from -95 to -270 mV (Li et al. 2015) and are influenced by chemical and physical factors such as pK_a , stereochemistry, thermodynamics and mechanical force.

pK_a

The thiol group of cysteine is subjected to deprotonation (loss of H^+) to form a thiolate anion that is more nucleophilic than a thiol. The ratio of thiol to thiolate in solution is dependent on the pH. A decrease in pK_a favours deprotonation and increases the fraction of the attacking thiolate to promote thiol-

Fig. 1 Schematic of thiol-disulphide exchange as a S_N2 -type nucleophilic substitution mechanism. **a** Energy profile of a S_N2 reaction as a function of the reaction coordinate. E_a represents the activation energy, which determines the rate of reaction, and ΔH is the change of free enthalpy during the reaction, which indicates how favourable the reaction is thermodynamically. The activation barrier can be lowered by catalysis of oxidoreductases, which is denoted as E_{cat} . Mechanism of **b** inter- or **c** intra-molecular thiol disulphide exchange in cleaving an allosteric disulphide bond. For thiol-disulphide exchange to proceed, the thiolate ion and the substrate allosteric disulphide bond are required to align linearly to enable nucleophilic attack of one sulphur atom of the disulphide bond. Alignment of the sulphur atoms can be facilitated by change in the conformation as a consequence of protein-protein interaction, ligand binding, and/or mechanical force. This figure is adapted from Hogg and Nagy (Hogg 2013; Nagy 2013)

disulphide exchange. For a free and unperturbed cysteine, the pK_a for thiol-thiolate at equilibrium is 8.5. In a protein, the pK_a of a cysteine is influenced by residues in the vicinity which can be charged, polar or hydrophobic. Depending on its microenvironment, the pK_a can range from 3.3 to 12 (Roos et al. 2013). Accessibility of a cysteine to solvent also affects its pK_a . Based on > 1000 protein crystal structures, the calculated pK_a for a buried cysteine is ~ 9.5 , whereas the calculated pK_a for a surface exposed cysteine is 7.5, which is much lower than that of a buried cysteine but is closer to physiological pH hence more likely to become deprotonated (Marino and Gladyshev 2010).

Formation of hydrogen bonds with a thiol group as a result of solvent exposure is important to lowering the pK_a of the

reacting thiolate. Analysis of a large number of protein crystal structures by molecular dynamics simulations reveals that the more hydrogen bonds are established with the sulphur atom, the lower the pK_a becomes, thus stabilising a thiolate anion for nucleophilic attack on disulphide bond for thiol-disulphide exchange (Roos et al. 2013). Sulphur-hydrogen bonds are not limited to interactions with polar or charged residues but can also be formed between sulphur and C α -H of the protein backbone as well as C-H moiety in the phenyl ring from aromatic residues.

Stereochemistry

Geometry and steric factors play important roles in the kinetics of thiol-disulphide exchange reaction. Since a linear conformation of the three sulphur atoms is essential, re-orientation of their molecular orbits may be necessary for S_N2 reaction to proceed. The degree of freedom of the sulphur atoms is limited by the conformational strain from the tertiary or quaternary structure of a protein (Nagy 2013). Other steric factors such as the presence of bulky functional groups in the reaction centre may hinder the access of the attacking thiolate, but could also facilitate the reaction by stabilising the binding of certain substrates. Positively charged residues near the reaction centre can stabilise the thiolate anion, while negatively charged residues can destabilise it (Wu et al. 2011).

Formation of disulphide bond can sometimes impose conformational strain in a protein. These pre-stressed disulphide bonds are usually more labile, and their reduction favours proteins to adopt conformations of lower energy state. Disulphide strains are therefore useful to define the reactivity of disulphide bonds, which will be discussed in the next section.

Thermodynamics

Thiol-disulphide exchange can occur in an intramolecular or intermolecular fashion. While there is no chemical distinction between the reactions once a disulphide bond is formed, an intramolecular reaction is associated with less entropy for reduction since the system only requires to overcome the translational degrees of freedom (Jensen et al. 2009; Nagy 2013). Because the reacting thiolate and the substrate disulphide bond are within the same polypeptide chain, any factor that brings the reacting thiolate and disulphide bond close together with the right geometry will effectively increase their molarity, which favours the S_N2 reaction to proceed for disulphide bond formation (Jensen et al. 2009). On the other hand, formation of an intermolecular disulphide bond requires the thiolate anion and the substrate disulphide bond, which are residing separately on two molecules, to orient in a linear conformation. Therefore, for S_N2 reaction to proceed, it requires the loss of both translational and rotational degrees of freedom to

overcome the wider distribution of rotamers arising from the participating proteins (Nagy 2013). Residues near the reaction centre also play an important role in stabilising the intermediate state of reaction species thereby lowering the activation energy (Jensen et al. 2009).

Mechanical force

Thiol-disulphide exchange is largely depending on deprotonation of the attacking thiol group (lowering pK_a) and overcoming the activation barrier posed by steric hindrance. Mechanical force has been demonstrated to influence the S_N2 reaction by changing protein conformation thus exposing buried thiol groups to aqueous solution leading to formation of new hydrogen bonds that lowers their pK_a (Beedle et al. 2018). Mechanical force can also unfold protein conformation to enable reduction of disulphide bonds. Using atomic-force microscopy, mechanical force can unfold protein at a single molecular level, exposing cryptic disulphide bonds to be acted upon by reducing agent like dithiothreitol, and oxidoreductases such as thioredoxin and PDI (Kosuri et al. 2012; Liang and Fernandez 2011; Wiita et al. 2006; Wiita et al. 2007).

Mechanical force can also distort the geometry of substrate disulphide bond by twisting, bending or stretching the sulphur-sulphur bond thus facilitating the alignment of sulphur atoms in favour of thiol-disulphide exchange. It has been determined using quantum and molecular dynamics simulations that mechanical force can shorten or elongate disulphide bond length and alter the geometry and enthalpy of the bond. By applying force equivalent to 300 pN, the redox potentials of disulphide bonds can be increased by 50 mV making them more likely to be cleaved (Baldus and Gräter 2012).

It is worth noting here that among all known modifications of cysteine thiols, disulphide bond is the only type that is mechano-sensitive. In other words, mechano-chemical coupling of thiol-disulphide exchange enables disulphide bonds to function as molecular switches for mechanical signals in proteins that they reside.

Signatures of allosteric disulphide bonds

The emergence of allosteric disulphide bonds as regulatory switches redefines some disulphide bonds from structural to regulatory role. Cleavage of allosteric disulphide bonds can increase or decrease ligand affinity of a protein, alter the kinetics of substrate hydrolysis of an enzyme, change the susceptibility to proteolysis of a protein, or regulate oligomerisation of a protein (Table 1).

To better differentiate the different types of disulphide bonds, it is necessary to define what makes a disulphide bond as allosteric. Proteolytic cleavage site by proteases in a protein is generally governed by the identity of amino acids on each

Table 1 Experimentally validated allosteric disulphide bonds in proteins

Regulation	Protein	Disulphide bond	Disulphide conformation ^a	Reference
Ligand interaction	β2-glycoprotein I	288–326	−/+ RHhook	(Ioannou et al. 2010; Passam et al. 2010)
	β3 integrin	177–184	−/+ RHhook	(Passam et al. 2018)
	CD4	130–159	− RHstaple	(Matthias et al. 2002)
	CD44	81–101	− LHhook	(Kellett-Clarke et al. 2015)
	HIV gp120	126–196	− RHstaple	(Auwerx et al. 2009; Barbouche et al. 2003; Gallina et al. 2002; Ou and Silver 2006)
		296–331	− RHstaple	
		385–418	− RHstaple	
	Glycoprotein 1b	4–17	− RHstaple	(Li et al. 2019)
		209–248	− LHhook	
	Interleukin-4	46–99	+ LHSpiral	(Plugis et al. 2018)
	Interleukin receptor subunit gamma	160–209	− LHhook	(Metcalf et al. 2012)
	Thrombospondin-1	974–?		(Pimanda et al. 2002)
	Tissue Factor	186–209	− RHstaple	(Ahamed et al. 2006; Chen et al. 2006; Ioannou et al. 2010)
	Vitronectin	137–161	Structure undetermined	(Bowley et al. 2017)
		274–453		
Substrate hydrolysis	von Willebrand factor	1669–1670	+/- LHStaple	(Butera et al. 2018; Ravilious et al. 2012)
	Adenosine-5'-phosphosulfate kinase	86–113	− RHhook	(Ravilious et al. 2012)
	Arylsulfate sulfotransferase	418–424	− RHstaple	(Malojic and Glockshuber 2010)
	Botulinum neurotoxins	436–445	− RHstaple	(Swaminathan and Eswaramoorthy 2000)
	CgDapF	83–221	− LHhook	(Sagong and Kim 2017)
	Lon protease	617–691	+/- LHSpiral	(Nishii et al. 2015)
	Methionine aminopeptidase 2	228–448	− RHstaple	(Chiu et al. 2014b)
	Prolyl cis-trans isomerase, AtFKBP13	106–111	− RHstaple	(Gopalan et al. 2004)
	QueF	55–99	− LHhook	(Mohammad et al. 2017)
	Tryptase β	191–220	− RHhook	(Cook et al. 2013)
	Thiolase	88–378	Undetermined	(Kim et al. 2015)
	SAMHD1	341–350	−/+ RHhook	(Mauney et al. 2017)
	Transglutaminase 2	370–371	− RHstaple	(Jin et al. 2011)
		230–370	+ RHstaple	
Proteolysis	Angiotensinogen	18–138	−/+ RHhook	(Zhou et al. 2010)
	Factor XI	362–482	−/+ RHhook	(Giannakopoulos et al. 2012)
	MICA	202–259	−/+ LHhook	(Kaiser et al. 2007)
	Plasminogen	512–536	− LHhook	(Butera et al. 2014)
		462–541	−/+ RHhook	
Oligomerisation	CD4	130–159	− RHstaple	(Matthias et al. 2002)
	Ficolin-3	6–23	Structure undetermined	(Eriksson et al. 2019)
	LYVE-1	201–201	− RHstaple	(Banerji et al. 2016)
	Vascular endothelial growth factors C & D	156–165	− LHhook	(Chiu et al. 2014a)

^a Conformation of disulphide bonds in protein crystal structures can be determined using the online tool Disulphide Bond Analysis (<http://149.171.101.136/python/disulfideanalysis/search.html>)

side of the cleavage site (Hedstrom 2002). However, no conserved recognition motif was found among the known allosteric disulphide bonds. Rather, due to the chemistry and geometry of disulphide bonds, the conformation of disulphide bonds in proteins plays a significant role in determining

whether the bond will undergo thiol-disulphide exchange (Schmidt et al. 2006). The geometry of a disulphide bond is defined by five dihedral angles ($\chi_1, \chi_2, \chi_3, \chi_2', \chi_1'$) between the C α and C α' atoms (Fig. 2a). There are three basic types of disulphide bond, spirals, hooks or staples, classified according

to their χ_2 , χ_3 , and χ_2' angles. The disulphide bond is further characterised as right-handed (RH) or left-handed (LH) based on whether the χ_3 angle is positive or negative, respectively. Finally, a χ_2 angle between 150° and 180° , or -150° and -180° is considered to be trans+ or trans−, respectively (Schmidt et al. 2006). Based on the geometry of these planar angles, Schmidt et al. defined 20 possible conformations for disulphide bonds using unique disulphide bonds identified in X-ray structures (Fig. 2a). By measuring the distances between the sulphur atoms and $C\alpha$ and $C\alpha'$ atoms, the dihedral energy strain exerted on each disulphide bond can be calculated, which is an indicator of disulphide reactivity and stability (Schmidt et al. 2006).

All 20 types of disulphide bonds exist in proteins. Among them −LHspiral bond is the most prevalent conformation accounting for 24.8% of the crystal structures analysed (Fig. 2b). The −LHspiral conformation has the lowest dihedral energy strain (10.1 kJ mol^{-1}) and can be considered as a structural disulphide bond that enables a protein to adopt a stable conformation with the lowest possible energy level (Schmidt et al. 2006). On the other hand, a −RHstaple disulphide bond ($-18.1 \text{ kJ mol}^{-1}$) is characterised with high dihedral energy strain and short distance between the $C\alpha$ and $C\alpha'$ atoms, thus

making it thermodynamically unstable and reactive (Schmidt et al. 2006). This disulphide conformation is found to be enriched among the allosteric disulphide bonds that are experimentally validated (Table 1). +/− RHhook disulphide bond is also characterised with high diherdral energy strain and is enriched among oxidoreductases, which reflects conservation of the Cys-X-X-Cys catalytic motif in the thioredoxin fold (Pijning et al. 2018; Schmidt et al. 2006). More recently, Pijning et al. hypothesised that allosteric disulphide bonds are labile in nature to enable their regulatory function as molecular switches (Pijning et al. 2018). Based on this assumption, they compared 13,031 disulphide bonds from proteins with both reduced and oxidised X-ray crystal structures, and characterised the conformations of these labile disulphide bonds. Their study found that −RHstaple disulphide bonds are enriched among the labile disulphide bonds making −RHstaple a definitive signature for allosteric disulphide bonds.

Externally applied force can modify a disulphide bond reactivity, but it has also been demonstrated that internal force arising from the topological constraints of the protein structure can influence the reactivity of a disulphide bond. Molecular dynamics simulations of 700 protein structures indicate that −RHstaple and +/− RHhook disulphide bonds are highly pre-

Fig. 2 Classification of disulphide bonds. **a** Disulphide bonds can be classified based on the geometry of the five dihedral angles (χ_1 , χ_2 , χ_3 , χ_2' , χ_1') of the two cysteines. Twenty possible conformations are resulted using this classification system, and all can be found in protein X-ray structures. Examples of each conformation are shown. Figure adapted from Chiu and Hogg (Chiu and Hogg 2019). **b** The distribution of each disulphide bond conformation using 13,031 non-redundant disulphide bonds available in the Protein Database. The three allosteric conformations, −RHstaple, +/− RHhook and −LHhook, accounts for ~20% of all non-redundant disulphide bonds in the database. Data was adapted from

Pijning et al. (Pijning et al. 2018). **c** Internal strain imposed on a disulphide bond can be measured by the bond length (d) and the torsional angles (α_1 and α_2) between the two sulphur atoms. The stresses placed on allosteric conformations −RHstaple and +/− RHhook are significantly higher than the other 18 conformations, making them pre-stressed in nature. Tensile prestress, which comprises all force types involved in sulphur-sulphur bond is a relevant indicator of the bond reactivity. Data shown is the mean tensile force of all 20 conformations adapted from Zhou et al. (Zhou et al. 2014)

stressed among the 20 types of disulphide bonds (Fig. 2c), with enhanced susceptibility for reduction in a similar fashion as having externally applied force on a reaction (Zhou et al. 2014). For example, –RHstaple disulphide bonds are characterised with an internally stretched sulphur-sulphur bond and torsional angles (α_1 and α_2) giving rise in tensile force in the range of 250 pN, which is similar to the amount of external forces needed to accelerate disulphide cleavage reaction (Zhou et al. 2014).

A third conformation emerges for allosteric disulphide bond which is the –LHhook conformation. –LHhook shows similar internal strain as the other 17 non-allosteric bond types (Zhou et al. 2014) and why it is associated with allosteric function remains undetermined. One possibility is that the –LHhook conformation is better suited for intra-molecular or non-facilitated thiol-disulphide exchange. For instance, plasminogen, which is the zymogen of plasmin that involves in the dissolution of clot formation, consists of a –LHhook disulphide bond Cys512-Cys536 (PDB: 4DUR) that can be cleaved by the nearby Cys541 thiolate anion (Butera et al. 2014). Another example can be seen in vascular endothelial growth factors C (VEGFC), which can exist as inactive monomer or active dimer. Monomeric VEGFC has an intramolecular disulphide bond Cys135-Cys156 and an unpaired Cys165, which can deprotonate and attack the Cys135-Cys156 bond in another monomer. As a consequence, dimeric VEGFC consists of an intermolecular Cys156-Cys165 disulphide bond, which is in –LHhook conformation (Chiu et al. 2014a). It is plausible that the unpaired Cys135 in VEGFC dimer can initiate another nucleophilic attack of the intermolecular bond to reduce the protein into monomers.

More recently, disulphide bond rearrangement has also been proposed in mechano-chemical regulation of the muscle protein titin. Three cysteines which are in close proximity within the I67 and I69 domains of titin suggest the possibility of reshuffling of disulphide bonds by thiol-disulphide exchange (Giganti et al. 2018). Based on the crystal structure of I-band fragment (I65-I70 domains; PDB: 3B43), I67 domain consists of three reduced cysteines, while I69 consists of a disulphide bond Cys8347-Cys8409 which adopts a –LHspiral conformation that is considered as an inert structural disulphide bond. External force is probably essential to pre-stress the disulphide bond to align it with the unpaired Cys8398 for a non-facilitated S_N2 reaction.

Temporal and spatial regulation of allosteric disulphide bonds

Oxidoreductases are enzymes that consist of an active-site motif of dithiol/disulphide (Cys-X-X-Cys) to reduce or oxidise a substrate disulphide bond. Formation of a cyclic disulphide in the active motif of an oxidoreductase is

energetically favourable and thus enhances intermolecular reduction of substrate disulphide bond (Nagy 2013). The role of oxidoreductases in thiol-disulphide exchange is crucial since they can lower the activation energy required to form the transition state complex (Fig. 1a).

Thioredoxin is the smallest oxidoreductase that is highly conserved in the evolution across all three kingdoms of life (Eklund et al. 1991). All other oxidoreductases have evolved from thioredoxin and contain at least one thioredoxin-like domain in their structure. At least 29 oxidoreductases have been identified in human, which include the PDI family, thioredoxins, quiescin sulphhydryl oxidase (QSOX), glutaredoxins (GLRX), and gamma-interferon-inducible lysosomal thiol reductase (GILT) (Table 2) (Alon et al. 2010; Appenzeller-Herzog and Ellgaard 2008; Flaumenhaft and Furie 2016).

Reduction of a disulphide bond by an oxidoreductase can proceed only when their energetics are matched, that is an oxidoreductase with lower redox potential (more negative value) will only donate electrons to a substrate disulphide with higher redox potential (more positive value). For example, transglutaminase 2 (TG2) is an enzyme that converts glutamine residues to glutamate in protein substrates and is an autoantigen in celiac disease. TG2 has an allosteric disulphide Cys370-Cys371 which can be reduced by thioredoxin to switch on TG2 activity in the extracellular matrix but oxidised by ERp57 to turn off TG2 function (Jin et al. 2011; Plugis et al. 2017; Yi et al. 2018). Cys370-Cys371 has a redox potential of –184 mV (Jin et al. 2011) which is compatible for reduction by thioredoxin with a redox potential of –270 mV (Krause et al. 1991) but also for oxidation by ERp57 that has redox potentials of –167 mV and –156 mV for its two catalytic disulphides in *a* and *a'* domains, respectively (Frickel et al. 2004).

Oxidoreductases are secreted by endothelial cells, platelets and neutrophils. PDI, ERp5 and ERp57 are secreted and bound to cell surface proteins such as integrin $\alpha IIb\beta 3$ when platelets are activated (Schulman et al. 2016). Similarly, neutrophils also secrete PDI when stimulated by cytokines (Hahm et al. 2013). The coupling of oxidoreductase secretion to the activation of platelets and neutrophils provides temporal control of disulphide cleavage by these enzymes. It is also possible that the timing of their secretion is linked to the expression of specific substrates on cell surface that require regulation by their redox activity and thus restrict their cleavage activity to specific substrates spatially (Chiu and Hogg 2019).

An active site dithiol of an oxidoreductase can act on a substrate disulphide bond and itself becomes oxidised as a consequence. The reducing power of an oxidoreductase is therefore consumed and requires regeneration (Chiu and Hogg 2019). While intracellular oxidoreductases can be recycled by the thioredoxin reductase system or Ero1 activity using electrons from NADPH or glutathione, respectively

Table 2 Oxidoreductases that are expressed in human

Oxidoreductase	Name	Catalytic motifs ^a	Localisation ^a
Thioredoxin family	TXN1	CGPC	Cytosol, nucleus, secreted
	TXN2	CGPC	Mitochondria
PDI family	PDI (P4HB)	CGHC, CGHC	Endoplasmic reticulum, cell secreted
	PDIp (PDIA2)	CGHC, CTHC	Endoplasmic reticulum
	ERp57 (PDIA3)	CGHC, CGHC	Endoplasmic reticulum, secreted
	ERp72 (PDIA4)	CGHC, CGHC	Endoplasmic reticulum, secreted
	PDIr (PDIA5)	CSMC, CGHC, CPHC	Endoplasmic reticulum
	ERp5 (PDIA6)	CGHC, CGHC	Endoplasmic reticulum, secreted
	PDILT (PDIA7)	SKQS, CGHC, SKKC	Endoplasmic reticulum
	ERp27 (PDIA8)	N/A	Endoplasmic reticulum
	ERp29 (PDIA9)	N/A	Endoplasmic reticulum
	ERp44 (TXNDC4)	CGHC	Endoplasmic reticulum, secreted
	TMX (PDIA11)	CPAC	Endoplasmic reticulum, plasma membrane
	TMX2 (PDIA12)	SNDC	Localisation unconfirmed
	TMX3 (PDIA13)	CGHC	Endoplasmic reticulum, plasma membrane
	TMX4 (PDIA14)	CPSC	Membrane
	TMX5 (Putative)	CRFS	Undetermined
	ERp46 (TXNDC5, PDIA15)		Endoplasmic reticulum
	ERp18 (TXNDC12, PDIA16)	CGHC	Endoplasmic reticulum
	AGR2 (PDIA17)	CPHS	Endoplasmic reticulum, secreted
	AGR3 (PDIA18)	CQYS	Endoplasmic reticulum
	ERdj5 (DNAJC10)	CSHC, CPPC, CHPC, CGPC	Endoplasmic reticulum
QSOX	QSOX1	CGHC, CRDC, CSAC	Endoplasmic reticulum golgi apparatus, secreted
	QSOX2	CGHC, CKEC, CPAC	Golgi apparatus, nucleus, plasma membrane, secreted
Glutaredoxin	GLRX1	CPYC	Cytosol, secreted
	GLRX2	CSYP	Nucleus, mitochondria
	GLRX3	CGFS	Cytosol, nucleus
	GLRX5	CGFS	Nucleus, mitochondria
GILT	GILT	CGGC	Lysosomes, secreted

^a Catalytic motifs and localisation is curated by Uniprot (www.uniprot.org)

(Kanemura et al. 2016; Poole 2015), how they are regenerated in the extracellular matrix is unknown. It has been proposed that secreted oxidoreductases are used in single reduction events to enable tight control of their activity without the need of an endogenous inhibitor (Chiu and Hogg 2019). Interestingly, human QSOX consists of a thioredoxin domain at its N-terminus but also a sulphhydryl oxidase domain at its C-terminus that can oxidise a dithiol using oxygen molecules, making QSOX a unique oxidoreductase that is capable of self-regenerating in the extracellular milieu (Alon et al. 2012).

Mechano-redox control of protein function in the vasculature

Disulphide chemistry has been demonstrated to be essential in regulating the function of coagulation factors such as tissue factor, Factor XI and vitronectin to mediate clot formation and haemostasis. It has also been shown that shear force regulates platelet adhesion to form a clot (Ju et al. 2018; Nesbitt et al. 2009). However, the interplay of disulphide bond and mechanical force in regulating platelet adhesion has not been shown until recently. We demonstrated the first example of

Fig. 3 Mechano-redox regulation of haemostasis. Vessel wall injury exposes collagen fibres in the subendothelium. Elevated shear force as a consequence induces unfolding of vWF enabling its anchorage to collagen and also unravelling binding site in A1 domain for GP1b α to tether platelets to the site of injury. vWF-GP1b α interaction is subjected to control by shear force which is necessary to unfold the globular structure of vWF, and by the allosteric disulphide bond Cys1669-Cys1670 in the A2 domain. Reduction of this bond or glutathionylation of Cys1670 increases the affinity of A1-A2 interaction thus blocking GP1b α binding under shear force. For stable clot formation, captured platelets become

activated inducing a conformational change in the α IIb β 3 receptor from bent to extended form. The opening of the α IIb β 3 headpiece exposes ligand binding pocket for fibrinogen binding and crosslinking of platelets. The ligand affinity of α IIb β 3 is regulated by the allosteric disulphide bond Cys177-Cys184. Secreted vascular oxidoreductase ERp5 can cleave this disulphide bond under shear force which leads to disengagement of fibrinogen. Binding of vWF to α IIb β 3 via its C4 domain is also essential to promote stable platelet aggregation under high shear force. Fibrinogen polymerises to fibrin fibres and forms a shear-resistant clot

coupling between a disulphide reaction and force in regulating the adhesive function of platelets. Secreted oxidoreductases such as PDI, ERp5, ERp57 and ERp72 are essential in clot formation upon vascular injury, but their mechanism of control was unclear (Cho et al. 2008; Holbrook et al. 2012;

Passam et al. 2015; Schulman et al. 2016; Wang et al. 2013; Zhou et al. 2017). Integrin α IIb β 3 is the most abundant receptor on platelet surface. On resting platelets, inactive α IIb β 3 adopts a bent conformation burying the headpiece from ligand interaction. Upon activation by agonists, α IIb β 3

undergoes a conformational change from bent to extended form, exposing its headpiece that enables fibrinogen binding and crosslinking of platelets for their adhesion, aggregation and clot formation at the site of vessel injury (Fig. 3). We identified an allosteric disulphide bond Cys177-Cys184 in the $\beta 3$ subunit that controls ligand affinity of $\alpha \text{IIb}\beta 3$ and is subjected to cleavage by secreted platelet ERp5 (Passam et al. 2018). Fibrinogen interacts with $\alpha \text{IIb}\beta 3$ in a catch bond manner such that their association weakens when external force is applied. Intriguingly, ERp5 activity promotes dissociation of fibrinogen from $\alpha \text{IIb}\beta 3$ when force is applied indicating that cleavage of Cys177-Cys184 disulphide bond couples with force to enhance fibrinogen release. Cleavage of this disulphide bond on the platelet surface decreases platelet adhesion to immobilised fibrinogen resulting in significantly less platelet aggregation more evidently at high shear rates (3000 s^{-1}). Cys177-Cys184 bond adopts a $-/+$ RHook conformation, which is naturally pre-stressed with high reactivity. Yet, this bond is not cleaved by ERp5 under static condition unless in the presence of ligand or under shear force (Passam et al. 2018) suggesting that stretching of the disulphide bond by either ligand binding or mechanical force is necessary for ERp5 activity. Dis-engagement of fibrinogen from platelets during clot formation seems counter-intuitive from the pro-thrombotic function of ERp5 observed in animal models of thrombosis (Passam et al. 2015). A possible explanation, but remains to be shown, is that fibrinogen release from $\alpha \text{IIb}\beta 3$ promotes binding of other ligands such as von Willebrand Factor for more stable adhesion under high shear force.

Another protein that is subjected to mechano-redox regulation is von Willebrand Factor (vWF). vWF is a force-sensing protein in plasma that captures platelets under conditions of high shear. vWF binds to collagen and platelet receptors $\text{GP1b}\alpha$ and $\alpha \text{IIb}\beta 3$ at the site of vascular injury. vWF binds to $\text{GP1b}\alpha$ via its A1 domain to tether platelets at high shear rates. Quiescence vWF adopts a globular conformation, which becomes unfolded upon stretching by elevated shear force thus exposing the binding site for $\text{GP1b}\alpha$ (Fig. 3). How vWF senses force to regulate $\text{GP1b}\alpha$ binding was unclear until the recent characterisation of an allosteric disulphide switch in the adjacent A2 domain (Butera et al. 2018). Butera et al. demonstrated that the Cys1669-Cys1670 disulphide bond in the A2 domain regulates its affinity to the A1 domain. Reduction of this bond increases A2 binding to the A1 domain thus blocking vWF interaction with $\text{GP1b}\alpha$ under shear force. This A2 disulphide switch therefore couples with shear force to regulate the scaffolding function of vWF. More importantly, the redox state of this disulphide switch is dynamic, which is mostly reduced in healthy individuals but is significantly oxidised in heart failure patients receiving extracorporeal membrane oxygen support and thus may provide some explanation of the prothrombotic complications associated with this device. How the A2 disulphide

bond is reduced or becomes oxidised remains undermined (Butera et al. 2018). Cys1670, which can be S-glutathionylated, may be key in regulating the redox dynamics of this disulphide bond.

The regulation of vascular proteins by disulphide bonds has created new opportunities for intervention in vascular and immune disorders. In particular, targeting secreted oxidoreductases has been shown to be less toxic and more effective than targeting other blood coagulation factors. For example, PDI which cleaves two disulphide bonds in vitronectin to promote clot formation can be inhibited by a flavonoid compound known as rutin. Isoquercetin, the modified form of rutin with enhanced bioavailability, has been shown to effectively prevent venous thrombosis in patients with cancer with minimal side-effects (Jasuja et al. 2012; Zwicker et al. 2019). Similarly, a small inhibitor NP161, which selectively oxidises the catalytic thiols of thioredoxin, attenuates the activity of secreted TG2 from macrophages making it a potential treatment for celiac disease (DiRaimondo et al. 2014). Oxidation of thioredoxin by NP161 is considered to be irreversible since no known thiol recycling mechanism is available for thioredoxin in the extracellular space (Plugis et al. 2017). Importantly, these inhibitors are non-permeable to cell membrane and only block the activity of secreted oxidoreductases without affecting the functions of intracellular enzymes thus making them less toxic to cells.

Conclusion

Disulphide bonds can serve as redox switches that induce allosteric effect in proteins to regulate their functions. Their mechano-sensitive nature enables the coupling of force and redox events in regulating functions in the vasculature. Emerging evidence indicates that mechano-redox control of cell receptors or clotting factors is essential in haemostasis and is likely to implicate regulation of other biological processes that are subjected to fluid shear.

Author contribution JC wrote the review with FJP.

Compliance with ethical standards

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Conflict of interest Freda J. Passam declares that she has no conflict of interest. Joyce Chiu declares that she has no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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