Review

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Enzymatic control of cysteinyl thiol switches in proteins

Abstract: The spatiotemporal modification of specific cysteinyl residues in proteins has emerged as a novel concept in signal transduction. Such modifications alter the redox state of the cysteinyl thiol group, with implications for the structure and biological function of the protein. Regulatory cysteines are therefore classified as ‘thiol switches’. In this review we emphasize the relevance of enzymes for specific and efficient redox sensing, evaluate prerequisites and general properties of redox switches, and highlight mechanistic principles for toggling thiol switches. Moreover, we provide an overview of potential mechanisms for the initial formation of regulatory disulfide bonds. In brief, we address the three basic questions (i) what defines a thiol switch, (ii) which parameters confer signal specificity, and (iii) how are thiol switches oxidized?

Keywords: complementarity; disulfide; hydroperoxide; redox catalysis; redox regulation; signal transduction.

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Introduction: thiol switches need enzymes

Cellular signal transduction requires the posttranslational modification of proteins, for instance the phosphorylation of seryl, threonyl, or tyrosyl side chains. These modifications change the biological functions of the proteins and thus allow a fine-tuned, well-orchestrated response to various external and internal stimuli. Posttranslational modifications can also occur at selected cysteinyl side chains. These modifications alter the redox state of the thiol group with implications for the structure and function of the protein. Such regulatory cysteinyl residues are therefore classified as ‘thiol switches’. The redox modification of thiol switches is highly specific with respect to the stimulus, the modified proteins, and the microenvironment of the modified thiol groups (Ghezzi et al., 2005; Jones, 2006; Seth and Stamler, 2011; Hanschmann et al., 2013). Moreover, the number of cysteines and thiol switches correlates with the complexity of the organism, highlighting the importance for the development of specific signal transduction pathways throughout animal evolution (Miseta and Csutora, 2000).

The toggling of protein thiol switches has long been attributed to changes in the ‘cellular redox state’, i.e., changes in redox potentials of compounds like the glutathione-glutathione disulfide (GSH:GSSG) redox couple. The redox potential is a measure of the Gibb’s free energy, and thus describes how thermodynamically favorable a reaction is. Differences in redox potential or free energy, however, are insufficient to predict whether and how fast a reaction will actually occur. The reaction rate is determined by the activation energy, an energy barrier/maximum along the energy surface of a reaction that corresponds to the transition state of the reactant(s). Reaction rates can be calculated with the help of rate constants, which are a function of the frequency of reactant collisions, the activation energy and the temperature as described by the Arrhenius equation: $k=A\cdot e^{-\frac{E_a}{RT}}$ (with A being the pre- or frequency factor, $E_a$ the activation energy, R the universal gas constant, and T the temperature). In vivo, reaction rates are controlled by the activity of enzymes, biocatalysts that massively increase the velocity of specific reactions by lowering the activation energies and by facilitating successful collisions between reactants because of geometric constraints. As an example, NADPH is a thermodynamically favorable reductant of GSSG ($\Delta E^o=80$ mV; $\Delta G^o=-15.4$ kJ/mol).
However, this reaction does not occur under physiological conditions. Only in the presence of the enzyme glutathione reductase (GR), do rate constants and reaction velocities reach reasonable dimensions. The second order rate constant of hydrogen peroxide ($H_2O_2$) with free cysteine is approximately $k=2.9 \ M^{-1} \ s^{-1}$. With GSH, the rate constant $k$ equals approx. $0.87 \ M^{-1} \ s^{-1}$ (Winterbourn and Metodiewa, 1999), which is similar to the rate constant around 1.05 $M^{-1} \ s^{-1}$ for the N-terminal active site thiol of the thiol-disulfide oxidoreductase thioredoxin (Trx) (Kagan et al., 2009). In contrast to Trx, peroxiredoxins (Prx) are enzymes that have been optimized for the specific reduction of $H_2O_2$ and other hydroperoxides. The reaction rate of the so-called peroxidatic cysteinyl residue at the active site of Prx ranges from 3 to $10^7 \ M^{-1} \ s^{-1}$ and is therefore four to six orders of magnitude higher than for ‘regular’ cysteinyl residues (Trujillo et al., 2007; Kagan et al., 2009). From these examples we can conclude (i) in vivo, most $H_2O_2$ will react with the thiol groups of dedicated peroxidases and (ii) only enzymatic catalysis allows physiologically relevant reaction velocities. For more comprehensive discussions on this topic, we refer to some of the following reviews Flohé (2010); Flohé (2013); Berndt et al. (2014); Forman et al. (2014); Marinho et al. (2014).

Next to their control of reaction velocities, enzymes also account for the second characteristic of redox signaling: specificity. Various hypothesis-driven as well as proteomic studies demonstrated the specificity of posttranslational redox modifications, specifically with respect to the redox modification (e.g., disulfide formation vs. sulfenylation), to the stimulus, to the oxidants and reductants, to the subcellular location and time point, to the protein modified, and to the cysteinyl residues that are the subject to oxidation and reduction, see for instance: Giustarini et al. (2005); Hammell-Pamment et al. (2005); Tannenbaum and White (2006); Foster et al. (2009); Forman et al. (2010); Seth and Stamlar (2011); Martinez-Ruiz et al. (2013); Balsera et al. (2014). Some of these residues may be allosteric thiol-disulfide switches (see below) that evolved to directly react with specific redox agents. The majority of thiol switches, however, most likely depends on the action of distinct enzymes. The glutathionylation and de glutathionylation of proteins, for instance, is specifically catalyzed by glutaredoxins (Grx) (Gravina and Mieyal, 1993; Gallogly and Mieyal, 2007; Berndt et al., 2008; Deponte, 2013). Although we are only beginning to understand the molecular mechanisms for redox signaling, especially in the case of targeted thiol oxidation, the reaction specificity, the spatiotemporal control, and the rate constants of redox signaling events can be only explained through enzymatic catalysis: thiol switches need enzymes.

### Categories of cysteine residues and their role as redox switches

Cysteinyl residues in proteins can be categorized based on a variety of interdependent parameters. These parameters include (i) the redox state of the sulfur atom, (ii) the molecular geometry of the cysteinyl residue with its bond lengths and angles, (iii) the accessibility and environment of the residue within a protein, and (iv) the properties as an acid/base or as a nucleophile/electrophile. All these parameters determine the reactivity and therefore affect whether a cysteine has a structural role, acts as a catalyst, or as a redox switch. So what are the properties of a good redox switch and can we predict redox switches based on these parameters?

(i) The versatile redox states of the sulfur atom in cysteinyl residues comprise thiols, thyl radicals, disulfides, sulfinic acids, sulfenic acids, and sulfonic acids (Figure 1A). Thiols and thyl radicals can be essential for enzyme catalysis, for example, to generate deoxyri bonucleotides with the help of ribonucleotide reductases (Jordan and Reichard, 1998; Nordlund and Reichard, 2006). Protein thiols also form intra- or intermolecular disulfide bonds. These can be classified as catalytic or structural disulfides based on their half-life. Short-lived disulfides are usually involved in catalytic redox cycles and are therefore formed and broken within milliseconds or seconds. Such catalytic cysteines are, e.g., found in many proteins of the thioredoxin superfamily (Deponte, 2013). In contrast, structural disulfides are formed under partially oxidizing conditions in the periplasm of Gram-negative bacteria or in the endoplasmic reticulum and the mitochondrial intermembrane space of eukaryotes (Deponte and Hell, 2009; Endo et al., 2010; Kodali and Thorpe, 2010; Codding et al., 2012; Oka and Bulleid, 2013; Hatahet et al., 2014; Kojer and Riemer, 2014). Once formed, such structural disulfides usually stabilize a protein in a defined tertiary or quaternary structure until the protein is degraded. However, selected structurally relevant disulfide bonds of secreted proteins were shown to be susceptible to reduction, which can result in altered protein conformations and functions (Hogg, 2003; Butera et al., 2014). Philip Hogg therefore introduced the term ‘allosteric disulfides’ to discriminate these bonds from classical structural and catalytic disulfides (Schmidt et al., 2006; Butera et al., 2014). Other non-catalytic and
non-structural disulfides with intermediate half-lives and regulatory functions are also found in the cytoplasm of bacteria and in reducing subcellular compartments of eukaryotes (Zheng et al., 1998; Aslund et al., 1999; Jakob et al., 1999; Delaunay et al., 2002; Jarvis et al., 2012; Sobotta et al., 2015; Nishii et al., 2015). Such redox switches include intramolecular disulfide bonds as well as intermolecular disulfide bonds between proteins or between a protein and a low molecular weight compound such as glutathione (Mieyal et al., 2008; Brigelius-Flohé and Flohé, 2011; Deponte, 2013). Sulfenic acids, which are an important source for disulfides, can be formed as reaction intermediates during catalysis. For example, the peroxidatic cysteinyl residues of Prx or of cysteine-containing glutathione peroxidases (GPx) are oxidized to a sulfenic acid upon reduction of a hydroperoxide substrate (Flohé et al., 2011; Klomsiri et al., 2011; Deponte, 2013). The sulfenic acids of Prx and GPx can subsequently form disulfide bonds with other peroxidase subunits or thiols or can be further oxidized to the sulfenic and sulfonic acid state (Figure 1A). Both modifications in Prx, the formation of intra- or intermolecular disulfide bonds (Jang et al., 2004; Brigelius-Flohé and Flohé, 2011; Chae et al., 2012) as well as the ‘over-oxidation’ (Wood et al., 2003; Klomsiri et al., 2011), are discussed as key events in redox signaling. Hence, the cysteinyl residue at the active site of Prx is a catalytic cysteine and a redox switch as outlined below. In summary, there are numerous functions for the different redox states of cysteinyl residues, and the redox state alone is unsuited to define a redox switch. Moreover, in order to act as a redox switch, a certain redox state of a cysteinyl residue has to be stable enough to allow its enrichment under steady-state conditions. Whether a redox switch can be turned off again depends on the stability of the resulting redox species and the enzyme repertoire. For example, sulfenic acids in certain Prx-isoforms can be regenerated in an ATP-dependent reaction that is catalyzed by sulfiredoxin (Figure 1A) (Biteau et al., 2003; Baek et al., 2012), but this enzyme appears to be absent in many organisms.

(ii) The molecular geometry of cysteinyl residues and disulfide bonds includes three classical structural parameters: bond lengths, bond angles between three atoms and dihedral angles between three bonds (Figure 1B). The geometry directly affects the reactivity as demonstrated for the Trx-catalyzed reduction of disulfides (Wiita et al., 2007). A systematic classification of the five dihedral angles between the Cα atoms of disulfide-bonded cysteines in annotated protein structures yielded 20 distinct disulfide configurations (Schmidt et al., 2006). Among these configurations, allosteric disulfides were predominantly found to have a minus right-handed staple bond (-RHStaple) (Schmidt et al., 2006), although more recent analyses revealed some exceptions to this rule (Butera et al., 2014). A minus left-handed spiral configuration (-LHSpiral) was found for many structural disulfide bonds, and catalytic disulfides in oxidoreductases often have a plus/minus right-handed hook configuration (+/-RHHook) (Schmidt et al., 2006).
The length of the disulfide bond and the α angles between the two Cβ-S-S bonds (Figure 1B) were recently considered in molecular dynamics simulations, suggesting that the allosteric -RHStaple and catalytic +/-RHHook disulfides are ‘pre-stressed’ because of bent α angles and a stretched disulfide bond (Zhou et al., 2014). Even though these tools will be helpful for the identification of candidate allosteric disulfides, the analyzed geometric parameters are still insufficient to explain or reliably predict whether a cysteine really acts as a redox switch.

(iii+iv) The reactivity and properties of a cysteiny1 residue are determined by the accessibility, the position and the environment of the residue within a protein. For example, the -RHStaple bond of allosteric disulfides is often found as a crosslink between antiparallel β-strands (Schmidt et al., 2006). This secondary structure presumably contributes to an activation of the disulfide bond (Zhou et al., 2014) based on a correlation between a stressed molecule geometry and an increase of the redox potential (Baldus and Grater, 2012). The reactivity of catalytic cysteines, e.g., in enzymes of the thioredoxin superfamily, is analogously influenced by the microenvironment that is generated by neighboring residues (Roos et al., 2009; Hall et al., 2011; Deponte, 2013; Lillig and Berndt, 2013; Van Laer et al., 2014). For example, lowering the pK_a of the thiol group with the help of proton acceptors or via ionic interactions with positively charged residues will not only affect the nucleophilicity of the cysteine but also make it a much better leaving group (Figure 1C) (Deponte, 2013; Nagy, 2013). Hence, the formation and the half-life of disulfides can be influenced by the pK_a value and the stability of the corresponding cysteine thiolates. Nevertheless, pK_a values and redox potentials are irrelevant for redox switches as long as the cysteiny1 side chain is inaccessible for an interacting redox agent. But even accessibility is not enough, because the kinetics of a productive interaction with a redox agent depend on a correct reaction geometry, in particular with regard to the transition state (Figure 1C) (Hall et al., 2010; Deponte, 2013; Nagy, 2013). Correct reaction geometries, together with complementary surfaces for signal molecules and interacting partners, are therefore crucial parameters for the specificity of thiol switches.

Taken together, redox switches should be accessible, have intermediate half-lives and sufficient reactivity. These parameters as well as the specificity of thiol switches are determined by the protein environment, resulting in activated molecular geometries as well as appropriate redox potentials, pK_a values and complementary surfaces. There are two major dilemmas for the prediction of redox switches. First, redox switching is often coupled to significant conformational changes (Choi et al., 2001; Jang et al., 2004; Wood et al., 2004; Jonsson et al., 2009; Hall et al., 2011; Nishii et al., 2015) and it is therefore difficult to predict the properties of a specific residue in the absence of structures that reflect the whole reaction pathway. Second, even for proteins that are structurally and functionally well-characterized, we often do not really understand the complex cysteine interactions at an atomistic level. Future studies will not only have to employ a quantum mechanical approach to precisely characterize the reactivity of the sulfur atoms but will also have to take into account the protein environment and its alteration during signal sensing and transduction.

Enzymatic mechanisms to flip a switch

The majority of enzymes that catalyze thiol-disulfide exchange reactions belong to the thioredoxin superfamily of proteins, i.e. Trx and Grx. These redoxins rely on cysteiny1 residues in a Cys-X-X-Cys/Ser active site motif. Trx and most of the Grx that contain both active site cysteiny1 residues reduce protein disulfides using a so-called dithiol mechanism. For recent summaries on this topic, see for instance (Lillig and Holmgren, 2007; Deponte, 2013; Hanschmann et al., 2013). The first step is a nucleophilic attack of the N-terminal cysteiny1 residue of the redoxin in its deprotonated thiolate form on the target disulfide (see also Figure 1C), resulting in an intermolecular disulfide between the N-terminal cysteiny1 residue and the attacked target cysteiny1 side chain. This intermediate disulfide is immediately attacked by the C-terminal thiolate of the redoxin active site, releasing the reduced target dithiol and leaving a disulfide at the active site of the redoxin. The Trx disulfide is subsequently reduced in a very similar thiol-disulfide exchange reaction by thioredoxin reductase (TrxR), whereas the Grx disulfide is reduced in two steps by GSH. In the first step a mixed disulfide between GSH and the N-terminal active site cysteiny1 residue of Grx is formed. In the second step the glutathionylated Grx is reduced by a second molecule of GSH, yielding reduced Grx and GSSG. According to the nomenclature of Cleland (Cleland, 1963), who also described dithiothreitol as thiol reductant (Cleland, 1964), Trx follow a ping-pong bi-bi reaction sequence whereas the Grx dithiol mechanism corresponds to an uni-uni-bi-uni ping-pong reaction sequence. Grx also catalyze the reduction of protein cysteiny1-glutathione...
mixed disulfides (deglutathionylation) using a so-called monothiol mechanism that only requires the N-terminal active site cysteinyl residue (Shelton et al., 2005; Gallogly and Mieyal, 2007; Lillig et al., 2008; Mesecke et al., 2008; Deponte, 2013). For the monothiol mechanism the N-terminal Grx cysteinyl thiolate attacks the mixed disulfide, yielding the reduced target protein and glutathionylated Grx. This intermediate, similar to the dithiol mechanism, is reduced by a second GSH molecule. Thus, in the monothiol mechanism, Grx follow a ping-pong bi-bi reaction sequence. Generally, all of the above mentioned reaction sequences are reversible (Figure 1C). It was thus proposed that proteins of the Trx superfamily may also specifically oxidize protein thiols, as demonstrated during oxidative protein folding for the Trx family members DsbA or protein disulfide isomerase (Ito and Inaba, 2008; Oka and Bulleid, 2013; Hatahet et al., 2014). However, experimental evidence for this hypothesis outside the bacterial periplasm and the eukaryotic endoplasmic reticulum is sparse. Thiol-disulfide exchange reactions with analogous mechanisms are furthermore catalyzed by enzymes with alternative protein folds, i.e., (i) GR and TrxR, (ii) Mia40, which catalyzes the oxidative protein folding in the intermembrane space of mitochondria, or (iii) enzymes of the Erv/QSOX family as reviewed (Fass, 2008; Deponte and Hell, 2009; Endo et al., 2010; Kodali and Thorpe, 2010; Kojer and Riemer, 2014).

Although Trx and Grx show a rather broad range of redox activities in vitro, they appear to have distinct substrate specificities in vivo. The basis and underlying mechanisms for this specificity, which is key to the understanding of thiol switches, are by no means clear. Some insights into the interaction of Trx family proteins with target proteins came from the structural analysis of the Prx-Grx hybrid protein from the proteobacterium M. Haemophilus influenzae (Figure 2B). Cytosolic human Trx1 and mitochondrial human Trx2 both show a similar charge distribution pattern and dipole character, i.e. a strongly negatively charged surface, independent of their redox state (Figure 2D–G). In contrast, cytosolic human Grx1 (Figure 2H) and, to a lesser extent, the cytosolic and mitochondrial human Grx2 (Figure 2I) both display a positively charged surface, which is presumably beneficial for the interaction of these proteins with the negatively charged carboxylate groups of glutathione (as shown on the surface of Grx2 in Figure 2I). Notably, E. coli Trx1 and Grx1 not only exhibit a similar substrate specificity, e.g. for ribonucleotide reductase or phosphoadenylyl sulfate reductase (Laurent et al., 1964; Holmgren, 1979; Lillig et al., 1999), but also display a more similar, rather neutral charge distribution on their surfaces (Figure 2J,K).

The relevance of complementary protein surfaces is further supported by a number of excellent structural studies on redoxins that were trapped as mixed disulfide intermediates with peptides corresponding to stretches of...
Electron sources and sinks for the formation of thiols and disulfides

How are protein thiols and disulfides initially formed in vivo? What are the final electron donors and acceptors? Protein disulfides are reduced with the help of NADPH, which is regenerated as universal electron donor either from glucose via the pentose phosphate pathway or via alternative pathways from 10-formyl-tetrahydrofolate, glutamate, aspartate and malate (Fan et al., 2014). As reviewed recently, there are two major pathways that link the NADPH:NADP⁺ pool with the protein thiol-disulfide pool in eukaryotes and many prokaryotes (Deponte, 2013). The flavoenzymes TrxR and GR act as electron relays. They initially transfer the electrons from NADPH to their FAD cofactor and from there to an internal disulfide. Reduced TrxR and GR subsequently catalyze thiol-disulfide exchange reactions with Trx and GSSG. The reduced adapter thiols Trx and GSH interact with a variety of target proteins such as ribonucleotide reductase and Grx (Figure 3A). Even though some eukaryotes and many prokaryotes have alternative adapter thiols and altered flavoenzyme relays (Manta et al., 2013; Van Laer et al., 2013), the biochemical principles are the same. But how are protein disulfides generated? Numerous thiol switches have been characterized in essentially all classes of organisms. At least in some cases, conditions have been described under which these switches are operated. However, except for well-characterized oxidative protein folding pathways in the bacterial periplasm, the mitochondrial intermembrane space, and the endoplasmic reticulum (Deponte and Hell, 2009; Endo et al., 2010; Kodali and Thorpe, 2010; Coddington et al., 2012; Oka and Bulleid, 2013; Hatahet et al., 2014; Kojer and Riemer, 2014), almost nothing is known about the molecular mechanisms of thiol oxidation.

One of the most compelling candidates as source of the oxidizing equivalents is H₂O₂, which is enzymatically produced and a well-established second messenger (Massey, 1994; Reth, 2002; Stone and Yang, 2006; Imlay, 2008; Kodali and Thorpe, 2010). Under physiological conditions, thiols react with H₂O₂ or other hydroperoxides to sulfenic acids that are readily reduced by adjacent thiols leading to the formation of disulfides (Figure 3B). As outlined above, to qualify as a thiol switch, both the oxidized...
and reduced thiol groups should have intermediate half-lives and display sufficient reactivity. Most protein thiols, however, show little reactivity with hydroperoxides. In competition with highly abundant dedicated peroxidases, with rate constants that are four to six orders of magnitude higher, sufficient reaction rates and extents cannot be expected, unless this competition is excluded by a highly localized H$_2$O$_2$ production close to the target thiol or other mechanisms of compartmentalization. In favor of the direct operation of thiol switches by H$_2$O$_2$ are some proteomic studies that demonstrated the presence of distinct protein sulfenic acids as identified by trapping and labeling with specific dimedone derivates (Klomsiri et al., 2010). Some of these studies aimed at the identification of all proteins that could potentially form cysteinyl sulfenic acids, mostly by treatment of cells with exogenous H$_2$O$_2$. A few studies could, however, demonstrate the presence of sulfenic acid modifications formed under physiological (or pathophysiological) conditions. For instance, 193 proteins harboring sulfenic acid modifications were identified in HeLa cell cultures (Leonard et al., 2009), about 100 in Arabidopsis thaliana cell suspensions that were treated with H$_2$O$_2$ (Waszczak et al., 2014), and, during the establishment of Medicago truncatula-Sinorhizobium meliloti symbiosis, 44 and 65 modified proteins in roots and the symbiotic organ, respectively (Oger et al., 2012). According to the floodgate hypothesis by Wood et al., the ‘over-oxidation’ of the peroxidatic cysteinyl residue in Prx to sulfinic and sulfonic acids, and thus their inactivation, serves as a regulatory gate that permits higher levels of H$_2$O$_2$ during signal transduction (Wood et al., 2003). As appealing as this model is to explain cysteinyl sulfenic acid formation in vivo, in the absence of Prx, the reduction of H$_2$O$_2$ by GPx and a reaction with millimolar GSH are kinetically still the most likely events in many organisms.

If not directly by H$_2$O$_2$, how are thiol switches flipped to their disulfide setting otherwise? A true redox signaling mechanism requires a primary signaling molecule, a

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**Figure 3** Physiological sources for the initial formation of thiols and disulfides.

(A) NADPH-dependent generation of reduced Trx and GSH and subsequent thiol-disulfide exchange reactions. (B) Putative direct H$_2$O$_2$-dependent oxidation of cysteinyl residues. See text for details. (C) Hydroperoxide- and Orp1-dependent oxidation of Yap1 and analogous GPx/Prx-dependent oxidations of potential transducer candidates. (D) Putative accumulation of Grx/Trx disulfides because of metabolic disulfide generation via ribonucleotide reductase (RNR). (E) Flavoenzyme- or ubiquinon (UQ)-dependent oxidative protein folding and putative thiol switch oxidation. Initial/final electron donors are colored in blue and initial/final electron acceptors are colored in red.
sensor, transducer(s), effector(s), and termination reactions (Brigelius-Flohé and Flohé, 2011). One of the few examples for which the exact mechanisms was unraveled is the Orp1/Yap1 couple from yeast (Delaunay et al., 2002; Azevedo et al., 2003; Avery et al., 2004; also discussed in detail by Brigelius-Flohé and Flohé, 2011). The GPx homologue Orp1 acts as sensor for H$_2$O$_2$, and becomes itself oxidized at Cys36 to a sulfenic acid. This sulfenic cysteinyl residue is reduced by Yap1 leading ultimately to a disulfide in Yap1 (Figure 3C). Oxidized Yap1 acts as the transducer of the H$_2$O$_2$ signal and promotes the transcription of specific genes. Trx terminates the signal by reducing the disulfide bond in Yap1. Could this example serve as general blueprint for the operation of other thiol switches? Peroxidases such as GPx and Prx would be ideal sensors for hydroperoxides. Most of all, they are abundant and reach significant reaction constants and rates with specific substrates (Hall et al., 2010; Flohé et al., 2011; Forman et al., 2014). Hence, GPx and Prx could efficiently transfer the hydroperoxide signal via thiol-disulfide exchange reactions to transducer and/or effector molecules (Figure 3C) (Brigelius-Flohé and Flohé, 2011). Several other peroxidase redox relays were indeed found in budding and fission yeast after the discovery of the Orp1/Yap1 couple (Delaunay et al., 2002). For example, the peroxiredoxin Tpx1 was shown to act as a hydroperoxide sensor and to oxidize and activate the transducer Pap1, which is the fission yeast homologue of Yap1 (Bozoned et al., 2005; Vivancos et al., 2005). Furthermore, the peroxiredoxins Ahp1 and Prx1 in budding yeast were reported to oxidize the transcription factor Cad1 and mitochondrial Trx3, respectively (Iwai et al., 2010; Greetham et al., 2013). Examples of mammalian sensor/transducer couples were identified only recently (Jarvis et al., 2012; Sobotta et al., 2015). Human Prx1 was reported to oxidize and therefore activate apoptosis signaling kinase 1 (Jarvis et al., 2012), whereas human Prx2 oxidizes and functionally attenuates the transcription factor STAT3 (Sobotta et al., 2015). The exact structure-function relationships of the more or less well-characterized sensor/transducer couples Tpx1/Pap1, Ahp1/Cad1 and Prx1/Trx3 in yeast or Prx1/Ask1 and Prx2/STAT3 in mammals are so far poorly understood. This is also true for the relevance of other Trx- and Grx-isoforms. They can either serve as reductants for Prx, or depend on GSH, which is not only the electron donor for some GPx but also a modulator of Prx function (Chae et al., 2012).

Another potential source for disulfide formation is metabolic activity. DNA synthesis, for instance, requires the reduction of ribonucleotides to deoxyribonucleotides by ribonucleotide reductase. This reduction leads to the formation of a disulfide in the enzyme that is reduced by Trx and Grx (Jordan and Reichard, 1998; Nordlund and Reichard, 2006). The size of the human genome exceeds 3×10$^{10}$ base pairs. The doubling of the human genome in the S-phase of the cell cycle thus requires the synthesis of twice the number of dNTPs leading to the generation of more than 6×10$^{9}$ disulfides. The total number of proteins in a single cell, e.g., HeLa, can be estimated to be in the range of 3×10$^{9}$–6×10$^{10}$ (Zhao et al., 2008; Milo, 2013). Hence, on average, one doubling of the genome leads to the generation of roughly 1–20 disulfides per single protein. This estimation exemplifies the potential of metabolic disulfide generation for redox signaling, even without considering oxidative protein folding. As Trx and Grx are the reductants of the disulfides formed in ribonucleotide reductase (Zahedi Avval and Holmgren, 2009), it is well conceivable that these disulfides could be also transduced to thiol switches of target proteins, thus directly linking metabolic activity to redox signals (Figure 3D). Again, the relevance of a hypothetical reaction between oxidized Trx/Grx and another protein will of course depend on the competing kinetics, in particular, considering the highly efficient reduction of Trx and Grx by TrxR and GSH (Zahedi Avval and Holmgren, 2009; Du et al., 2012).

Recently, some flavin monooxygenases of the MICAL family have been demonstrated to specifically oxidize methionyl residues to methionyl-sulfoxides using molecular oxygen and NADPH as substrates. MICALs are transducer proteins of the Sema3A-PlexA signaling cascade, required for axon guidance during neurogenesis (Terman et al., 2002). Even though the exact mechanism of MICAL remains to be analyzed, its stereospecificity (Lee et al., 2013) suggests that the target protein is directly oxidized by a non-diffusible flavin hydroperoxide intermediate and not by diffusible H$_2$O$_2$. The flavin hydroperoxide could be formed in a direct reaction between molecular oxygen and the reduced flavin (FADH$_2$) as has been suggested or shown for other flavoenzymes (Massey, 1994). One of the oxygen atoms is then probably transferred to a nucleophilic substrate, such as a methionyl residue in β-actin (Hung et al., 2010; Lee et al., 2013). Flavoenzymes that catalyze the direct oxidation of thiols are found in a variety of metabolic pathways as reviewed previously (Deponte and Hell, 2009). Again, the flavoenzymes act as versatile electron relays between a thiol:disulfide pool and different non-thiol redox agents such cytochrome c$^{3+}$, O$_2$, or NAD$^+$. However, in contrast to NADPH-dependent reduction of disulfides, the flow of electrons is reversed and occurs from the thiol substrate to an internal disulfide of the flavoenzyme, yielding a substrate disulfide. After the thiol-disulfide exchange reaction, the reduced flavoenzyme
subsequently transfers the electrons to its FAD cofactor and from there to the oxidant (Figure 3E). For example, dihydrolipoamide dehydrogenases act as central subunits of α-keto acid dehydrogenases with NAD$^+$ as final electron acceptor, and members of the Ero1 and Erv families of sulfhydryl oxidases and electron transferases function in oxidative protein folding with molecular oxygen or cytochrome c$^{+}$ as electron acceptors (Bihlmayer et al., 2007; Fass, 2008; Deponte and Hell, 2009; Kodali and Thorpe, 2010; Eckers et al., 2013). The Erv module can also be part of a multidomain setup, for instance in the quiescin sulfhydryl oxidase (QSOX) family that also includes an additional thioredoxin domain and acts in the secretory pathway and extracellularly (Kodali and Thorpe, 2010; Coddings et al., 2012). Last but not least, in some thiol oxidation pathways, i.e., for DsbB-dependent oxidative protein folding in the bacterial periplasm, quinones replace the flavin as an electron relay (Figure 3E) (Kodali and Thorpe, 2010; Hatahet et al., 2014; Kojer and Riemer, 2014). Although a direct oxidation of a thiol switch by a flavoenzyme or quinone is an attractive option, such a reaction has not been proven to date.

In summary, the precise mechanisms of thiol switch oxidations in vivo are still one of the greatest mysteries in redox research. Unraveling these secrets in cell biology on a molecular level will be crucial for any future attempt to model redox-dependent signal transduction pathways as well as for the development of potential therapeutic strategies.

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