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Signalling networks in focus

Thiol-based redox signalling: Rust never sleeps

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ABSTRACT

Cysteine residues in proteins are covalently modified under conditions of oxidative and nitrosative stress by oxidation, nitrosation, glutathionylation and disulfide formation. Modifications induce conformational changes in substrate proteins, effecting signal cascades that evoke a biological response. A growing number of structures with modified cysteines are allowing a piecemeal understanding of the mechanistic aspects of these signalling pathways to emerge. Conformational changes upon conjugation of nitric oxide and glutathione are generally small and often accompanied by a local increase in protein disorder. Burial of nitric oxide is also apparent, which may increase the timeframe of signalling. Conformational changes upon disulfide formation/reduction range from the small to the spectacular. They include order/disorder transitions; oxidation of disulfides following expulsion of metals such as Zn; major reorganisation or "morphing" of portions of the polypeptide backbone; and changes in quaternary structure including domain swapping.

Signalling network facts

- Modification of cysteine residues by reactive oxygen and nitric oxide species or "thiol-based redox signalling" are emerging signalling pathways which may be as important in cellular signalling as protein phosphorylation.
- Cysteine modifications are often transitory, localized, and refractory to analysis. It is easier to detect the instigating reactive oxygen species than the modifications themselves.
- First characterized in plants, thiol-based redox signalling is known to operate in all three kingdoms and was likely crucial to establishment of life on earth and its adaptation to an oxygen-based environment.
- Unlike phosphorylation, there is no amplification of the signal through a hierarchy of targets. The signal can be scaled from the minute, by localization of reactive oxygen species and substrates, to mass action, by adjustment of the redox poise of the compartment enabling simultaneous modification of multiple substrates.
- Ramping of the response is possible because each substrate has an individual set point or redox potential at which it will respond. In phosphorylation the signal is amplified through several stages. In thiol-based redox signalling, different "alert states" are associated with increasing levels of reactive oxygen species. Additional pathways are recruited as reactive oxygen species levels escalate.
- As the stimulus acts directly on the substrate and does not need to be mediated by a protein, the response is fast.
- There is likely substantial crosstalk between (oxygen-based) phosphorylative cascades and thiol-based redox signalling. Modes identified include: initiation of phosphorylative cascades by presentation of phosphorylative sites (AKT2); potentiation of cascades by inhibition of phosphatases (PTPs); and release of latent kinases (ASK).

1. Introduction

Regulation of protein function via posttranslational modifications is a burgeoning area of interest underpinned by advances

Abbreviations: Arg, arginine; Cys, cysteine; ER, endoplasmic reticulum; Grx, glutaredoxin; GSH, glutathione; GSHR, glutathione reductase; GSSG, glutathione disulfide; GST, glutathione *S*-transferase; Lys, lysine; NO, nitric oxide; PDB, Protein Data Bank; PDI, protein disulfide isomerase; RMSD, root mean square deviation; ROS, reactive oxygen species; Ser, serine; SG, gamma sulfur; SOD1, superoxide dismutase; Trx, thioredoxin; TrxR, thioredoxin reductase; Thr, threonine; Tyr, tyrosine; YFP, Yellow Fluorescent Protein.

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in high throughput proteomics. Intracellular signalling mediated by reversible phosphorylation of serine (Ser), threonine (Thr) and tyrosine (Tyr) residues of proteins is the best characterized post translational process. More recently multiple modifications of arginine (Arg) and lysine (Lys) residues have come to the fore. For example, Lys can undergo methylation, acetylation and hydroxylation; and can also be covalently cross-linked to different proteins in ubiquitination and similar processes, as well as transglutamination (Begg et al., 2006). Modifications of Lys mediate diverse processes from epigenetics, to protein fate and structural processes. In addition to these two basic residues, another residue which undergoes multiple modifications but which has received considerably less attention is cysteine (Cys).

The diversity of modifications of Cys is extensive (Fig. 1) and crucial to sustainable life in the oxidizing environment of this planet (Wouters et al., 2010; Nagahara et al., 2009). Though important, Cys modifications are often transitory and refractory to analysis (Riederer, 2009). Interestingly, Cys has one of the shortest sidechains whereas Lys and Arg have among the longest, extending up to 6.5 Å (Lys) or 7.5 Å (Arg) from the polypeptide backbone. As a result, surface modifications of Arg and Lys are tethered far from the backbone, almost like labels on the protein. In contrast, modifications of Cys residues, and its isostere Ser, occur closer to the protein surface and are often accompanied by conformational changes of the backbone. In addition, Cys has the unique ability to form disulfide bonds, enabling some spectacular redox-driven conformational changes. Unlike transglutamination, the other common covalent protein crosslink between Lys and glutamine (Begg et al., 2006), formation of disulfide bonds between Cys pairs is potentially reversible.

Generation of reactive oxygen species (ROS) is a natural byproduct of many oxidative processes in the cell. Major sources of ROS include oxidative phosphorylation by mitochondria, and protein folding in the endoplasmic reticulum (ER). Sulfur atoms are natural targets for ROS because of their polarizible nature. For example, sequential oxidation of Cys yields sulfenic, sulfinic or sulfonic acids. More energy is required to reduce the higher derivatives, thus cells employ several molecular strategies to prevent their formation (Fig. 1). However, these pathways are more than merely redox homeostasis systems. The cell monitors fluxes of these molecules and effects biological processes in response to them such as the oxidative stress response. At the molecular level, how these signals are transduced by the protein substrate is only beginning to be understood.

2. Functions

2.1. Conformational changes upon conjugation of small molecules

In protein *S*-nitrosation also known as "nitrosylation" nitric oxide (NO) is conjugated to Cys. NO is produced endogeneously from L-Arg and oxygen by NO synthases, but its direct action is limited by its short one sec half-life in vivo, which limits the distance that NO can traverse. The time frame of its action is sustained by the Cys-containing substrates it modifies. NO modification was first noted because of its profound effect on vascular tone, a response mediated by regulation of cGMP production (Stamler et al., 1992). Multiple other substrates of NO have been identified, widening its role to tissue development, memory formation, and cell growth and death (Schreiter et al., 2007).

The rarity of NO modification in crystal structures (Table 1) in no way reflects its rarity in physiologic systems (Hao et al., 2006). Because of NO's redox activity, care must be taken to minimize X-ray exposure during structure solution. The structure of tuna myoglobin was solved using a rotating anode X-ray generator because synchrotron radiation completely removed the NO group, as did longer X-ray exposure times (Schreiter et al., 2007). NO adducts are reasonably stable when stored in the dark and in metal-free buffer (Schreiter et al., 2007).

Like phosphorylation of Ser residues, conformational changes induced by NO are quite subtle (Fig. 2). In thioredoxin 1 (Trx1) and myoglobin, the protein expands slightly. *S*-Nitrosation of Cys 10 in myoglobin (Schreiter et al., 2007) acts like a wedge, expanding the protein and perturbing three discrete portions of polypeptide chain. Burial of the NO group in Trx1 and haemoglobin may increase the signalling timeframe. Structural disorder may also be introduced: in tuna myoglobin, the NO group adopts two distinct conformations (Yi et al., 2005); in human haemoglobin, NO displaces the intrinsically disordered C-terminal dipeptide of the β subunit from a stabilizing binding pocket (Chan et al., 1998).

Glutathione (GSH), the most abundant low-molecular-weight thiol in the cell, can also form adducts with oxidized Cys in proteins. GSH is a tripeptide consisting of glutamine, Cys, and glycine that is generated as a redox buffer by the cell. The oxidized form, glutathione disulfide (GSSG), is generated by disulfide linkage of Cys residues from two GSH peptides. A 30- to 100-fold excess of reduced GSH over the oxidized form supports a reducing environment in the cytosol (Hwang et al., 1992). The ratio of GSH to GSSG is largely maintained by glutathione reductase (GSHR). At concentrations of 1–10 mM in the eukaryotic cytosol, GSH is the major initial cytosolic target of NO, yielding *S*-nitrosoglutathione. In general, GSH is removed from proteins by the thiol oxidoreductase glutaredoxin (Grx), which is in turn reduced by thioredoxin reductase (TrxR, Fig. 1).

Although there are around 80 solved structures with bound GSH, the molecule is likely to be covalently bound in around only 20 unique structures (Table 1). Most structures with non-covalently bound GSH are glutathione *S*-transferases (GSTs) which conjugate GSH to small electrophilic compounds to detoxify them. Although most GSTs bind GSH non-covalently, in bacterial β-GSTs, GSH *is* bound covalently (Rossjohn et al., 1998). β-GSTs suggest GSH's dual functions in detoxification and redox buffering may have a common origin: a hypothesis supported by homology between GSTs and Trx (Martin, 1995).

Most solved structures of proteins conjugated with GSH have Trx-like folds (group 1, Table 1). These include Grxs, Trx, β-GSTs and CLIC1, a protein that counter-balances intracellular ionic charge in response to excess electrons produced by membrane-bound NADPH oxidase under conditions of oxidative stress (Averaimo et al., 2010). In group 1 structures, two backbone hydrogen bonds bind GSH as an additional antiparallel strand (Bushweller et al., 1994). In group 2 structures GSH is bound to FAD-dependent enzymes: GSHR and T54 methyltransferase. In all these enzyme structures, where GSH is generally a substrate, the GSH is well-ordered and has multiple interactions with the enzyme along the length of the peptide.

Despite a lengthening list of proteins known to be regulated by covalent binding of GSH, only a single structure of carbonic anhydrase, which is oxidized in hepatocytes under conditions of oxidative stress, is available (Mallis et al., 2000). In contrast to the well-ordered binding in redox enzymes, GSH conjugation to carbonic anhydrase is a surface modification with little interaction between GSH and the protein, resulting in significant disorder of the GSH distal to the covalent bond. In this disordered state, GSH may act as a flipper, disrupting protein interactions or preventing aggregation. This function-by-disorder mechanism is also apparent in NO-conjugated structures.

Structures of GSH bound to Trx1 and CLIC1 are intermediate between these two extremes. In Trx1, which generally reduces disulfide bonds in proteins rather than GSH adducts, there are fewer interactions. In CLIC1, the partially stable glu-