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REVIEW ARTICLE

Protein oxidation: role in signalling and detection by mass spectrometry

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Abstract Proteins can undergo a wide variety of oxidative post-translational modifications (oxPTM); while reversible modifications are thought to be relevant in physiological processes, non-reversible oxPTM may contribute to pathological situations and disease. The oxidant is also important in determining the type of oxPTM, such as oxidation, chlorination or nitration. The best characterized oxPTMs involved in signalling modulation are partial oxidations of cysteine to disulfide, glutathionylated or sulfenic acid forms that can be reversed by thiol reductants. Proline hydroxylation in HIF signalling is also quite well characterized, and there is increasing evidence that specific oxidations of methionine and tyrosine may have some biological roles. For some proteins regulated by cysteine oxidation, the residues and molecular mechanism involved have been extensively studied and are well understood, such as the protein tyrosine phosphatase PTP1B and MAP3 kinase ASK1, as well as transcription factor complex Keap1–Nrf2. The advances in understanding of the role oxPTMs in signalling have been facilitated by advances in analytical technology, in particular tandem mass spectrometry techniques. Combinations of peptide sequencing by collisionally induced dissociation and precursor ion scanning or neutral loss to select for specific oxPTMs have proved very useful for identifying oxidatively modified proteins and mapping the sites of oxidation. The development of specific labelling and enrichment procedures for S-nitrosylation or disulfide formation has proved invaluable, and there is ongoing work to establish analogous methods for detection of nitrotyrosine and other modifications.

Keywords Redox signalling · Nitrotyrosine · Oxidative stress · Precursor ion scanning · Cysteine oxidation

Abbreviations

cICAT	Cleavable isotope coded affinity tag
CID	Collision induced dissociation
Duox	Dual oxidases
ECD	Electron capture dissociation
eNOS	Endothelial nitric oxide synthases
ESI	Electrospray ionization
FIH	Factor inhibiting HIF
HIF	Hypoxia inducible factor
iNOS	Inducible nitric oxide synthases
iTRAQ	Isobaric tag for relative and absolute quantification
MALDI	Matrix assisted laser desorption/ionization
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
Nox	NADPH oxidase
oxPTM	oxidative post-translational modifications
Phox	Phagocyte oxidase
PHD	Proline hydroxylation domain

Review invited for a special issue of Amino Acids dedicated to *Amino acid and protein modification by oxygen and nitrogen species.*

This article is part of the Special Issue on Redoxomics of proteins and amino acids.

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PTP	Protein tyrosine phosphatase
SNO	S-nitrosothiol
Trx	Thioredoxin

Protein oxidation and oxidants

It has now been established quite firmly that oxidative post-translational modifications are not simply damaging to the protein and deleterious to the cell, but that certain modifications under appropriate conditions constitute important signalling mechanisms. This has greatly boosted the interest in this field. Redox signalling is involved both in response to stress situations, such as infection and inflammation, and in normal physiological control of cell behaviour (Droge 2002). A variety of partially reduced oxygen species [superoxide (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH·)], reactive nitrogen species [nitric oxide (NO), nitrogen dioxide (N_2O) and peroxynitrite ($ONOO^-$)] and reactive chlorine species (e.g. hypochlorous acid, HOCl) can be produced by enzymatic and chemical reactions during phagocyte activation in inflammation, most of which are capable of reacting with the side chains in proteins. Moreover, superoxide, hydrogen peroxide and nitric oxide can be produced during normal metabolism. Mitochondrial electron leakage during respiration, redox cycling enzymes such as cytochrome P450, xanthine oxidase and metabolite autoxidation can all result in superoxide formation (Sauer 2001). More importantly for redox signalling, many cells contain NADPH oxidases (Nox) or dual oxidases (Duox); these are functionally linked to receptors, and produce superoxide following receptor-mediated responses to certain growth factors such as EGF (Brown and Griendling 2009; Lambeth 2002). Nitric oxide is produced by nitric oxide synthases (NOS): the endothelial form, eNOS, is constitutively active and involved in signaling via soluble guanylate cyclase, while the inducible phagocyte form, iNOS, is activated in response to infection and has a higher output (Ignarro 1990). Oxidant production by Noxs and eNOS can be regarded as physiological processes, while oxidant production in phagocytic cells via Phox or iNOS relates more to a pathological situation. Protein oxidation also plays a key role in sensing of oxygen levels through the iron/ascorbate/2-oxoglutarate-dependent prolyl hydroxylases (PHD 1–3) (Kaelin and Ratcliffe 2008) and asparaginyl hyroxylase (FIH) (Lando et al. 2002; Mahon et al. 2001), which catalytically hydroxylate their respective amino acids using molecular oxygen as a substrate.

As most of the oxidants mentioned above are capable of oxidizing biomolecules, it is not necessarily obvious which are actually responsible for the oxPTMs involving in redox signalling. S-nitrosylation of proteins is well established as a mechanism for regulating protein activity and has received considerable interest (Stamler et al. 1992); it clearly indicates the involvement of reactive derivatives of nitric oxide, although it is accepted that these may be ones formed downstream of NO, such as peroxynitrite, nitrogen dioxide (NO_2) or dinitrogen trioxide (N_2O_3) (Gaston et al. 2006; Hess et al. 2005). However, peroxynitrite can cause oxidation as well as nitration or nitrosylation of proteins, through its ability to generate, nitrogen dioxide, hydroxyl and carbonyl radicals (Radi 2004). In terms of partially reduced oxygen species, hydrogen peroxide is the compound most often discussed, and to which signaling functions are commonly attributed. However, although it has a high (positive) reduction potential, many of the reactions in which it is involved also have high activation energies, so although they are favourable, they are also slow (Winterbourn and Hampton 2008). There is some debate about whether hydrogen peroxide would be able to react with signaling proteins at a sufficient rate to contribute to signaling processes; however, its reactions with glutathione and other antioxidants are also relatively slow and therefore would not preclude reactions with protein side chains. This has led to the suggestion that hydrogen peroxide may not react directly with the target protein, but instead the oxidative signal is transduced by redox sensors, possibly peroxiredoxins (Janssen-Heininger et al. 2008; Winterbourn and Hampton 2008). Alternatively, redox signaling proteins may response to the local thiol–disulfide balance. In general, it is considered that highly reactive oxidants such as hydroxyl radical and hypochlorous acid are unlikely to contribute to signaling as they are insufficiently discriminating, and would be unable to achieve the selective modification of signaling proteins. For example, hypochlorous acid is able to react with both thiol and thiolate forms of cysteine side chains (Peskin and Winterbourn 2001), so its reactivity is not dependent on the p*K*a of the thiol group within the protein.

The aim of this review is to illustrate the growing understanding of protein oxidation in redox signalling using examples of the best characterized proteins involved, and to describe the advances in technology, especially mass spectrometry, that allow investigation of these oxPTMs.

Cysteine oxidations are common oxPTMs

The sulfur-containing residues cysteine and methionine are generally the most susceptible to oxidation, owing to their high redox reactivity (Davies et al. 1987; Davies 2005; Radi et al. 1991; Vogt 1995). If cysteine is a catalytic

residue in the active site, its oxidation will influence directly the activity of the enzyme, but even if it is not directly involved in catalysis, oxidation will change the local conformation and potentially alter the protein's activity. Cysteine oxidations can also affect protein–protein interaction, protein degradation, and other post-translational modifications. Cysteine can be oxidized to several products: disulfides, sulfenate ($–SO^-$), sulfinate ($–SO_2^-$), sulfonate ($–SO_3^-$) or sulfenamide (–SNR); mixed disulfides with glutathione may also occur (glutathionylation), and reactive nitrogen species can result in S-nitrosylation (–SNO). In most proteins, formation of sulfinate or sulfonate forms is an irreversible modification, although in peroxiredoxins over-oxidation to the sulfinate can be recovered by sulfiredoxin; this enzyme was only discovered relatively recently (Biteau et al. 2003) and as yet its activity is thought to be limited to reduction of cysteine oxidations in 2-Cys peroxiredoxins (Lei et al. 2008; Rhee et al. 2005).

Not all cysteines are equally reactive, as the thiolate ($–S^-$) form is more reactive and is oxidized considerably faster than the thiol (–SH) form, so the potential for oxidation is determined by the p*K*a. In free cysteine the p*K*a of the thiol is approximately 8.2, but in proteins the local environment influences the ionizability of the thiol; the presence of basic groups that can stabilize the thiolate facilitate deprotonation (Forman et al. 2004; Winterbourn and Hampton 2008). In peroxiredoxins and protein tyrosine kinases, the p*K*a of active site cysteine thiol groups may be as low as 4.5. This could logically contribute to the specificity in protein oxidation, a prerequisite for signalling, as only proteins containing cysteines with p*K*as less than 6.5 are likely to undergo significant oxidation. However, a comparison of the p*K*a values and rates of reaction of several proteins with hydrogen peroxide shows that, while for low molecular weight thiols such as N-acetylcystine and glutathione there is a clear relationship between p*K*a and rate constant, in proteins the situation is more complex. In particular, peroxiredoxins have a rate constant 10^4–10^6-fold higher than other proteins with similar p*K*as, such as protein tyrosine phosphatases. For example, the rate constants for thioredoxin (p*K*a 6.5), PTP1B (p*K*a 5.4), and peroxiredoxins (pK_as 5–6) are 1.05, 20, and 1–4 $\times$ 10^7 M^{-1} s^{-1}, respectively (Denu and Tanner 1998; Goldman et al. 1995; Ogusucu et al. 2007; Parsonage et al. 2008; Peskin et al. 2007); reviewed by (Winterbourn and Hampton 2008). This suggests a low p*K*a is not sufficient to result in detectable oxidation and explain differential reactivity, at least for oxidation by hydrogen peroxide, and other factors such as cysteine accessibility may also be important. The same is likely to be true for nitrosylation of cysteines by RNS, and possibly by other oxidants.

Signalling regulation by sulfur-containing residues

Signalling proteins regulated by thiol oxidation

Redox-dependent behaviour has been reported for proteins involved in most aspects of cellular processes: metabolism, inflammatory responses, cell proliferation and differentiation, survival or apoptosis, and most signalling pathways can be modulated by cellular redox status. These effects depend on the activation, or more commonly, inactivation of specific proteins, usually by reversible cysteine oxidation. The literature on proteins redox-regulated by oxidation of cysteine residues is growing rapidly, along with our understanding of the molecular mechanisms involved in reversible thiol oxidation (Forman and Torres 2002; Janssen-Heininger et al. 2008; Rhee et al. 2005; Spickett et al. 2006; Winterbourn and Hampton 2008). For some proteins, or families of proteins, the relevant residues and chemical mechanism of the reversible redox cycle have been characterized. Examples of better characterized redox-regulated proteins include the transcription factors YAP-1, Oxy R/S, SoxR/S and NFκB, the protein tyrosine phosphatases SHP1/2 and PTP1B, peroxiredoxins, thioredoxin, caspases, sarcoplasmic reticulum Ca^{2+}-ATPase, and some components of the mitogen-activated protein kinase cascades such as MEKK1 and ASK1. The redox regulation of signalling pathways is complex, and often involves multiple mechanisms: for example protein tyrosine phosphatases are inactivated by oxidation, which causes increased levels of phosphorylated substrates and overall activation of kinase pathways, but additionally some kinases are activated by oxidative mechanisms (e.g. ASK1). This section will illustrate current understanding of some principles of thiol-dependent redox signalling using a few salient examples.

Some paradigms are now emerging in the mechanisms of reversible cysteine signalling, such as the thiolate–sulfenate cycle. Hydrogen peroxide reacts with a protein thiolate group to form the sulfenate, which subsequently often reacts with a thiol reductant (RSH), generating a mixed disulfide. The reductant is usually glutathione or thioredoxin; in the case of the former, the cysteine is glutathionylated. In the final step, the thiolate form of cysteine is regenerated by reaction of the mixed disulfide with a second RSH, releasing RSSR (Fig. 1). For a number of proteins, it has also been found that glutathionylation can occur directly by reaction of the protein thiolate with glutathione disulfide. An alternative mechanism of oxidation is by nitric oxide-derived species such as NO^+ or peroxynitrite; this causes S-nitrosylation to form the S-nitrosothiol (–SNO). The –SNO can be converted back to the cysteine thiol form by reaction with glutathione, or possibly other thiol reductants; the other product of this

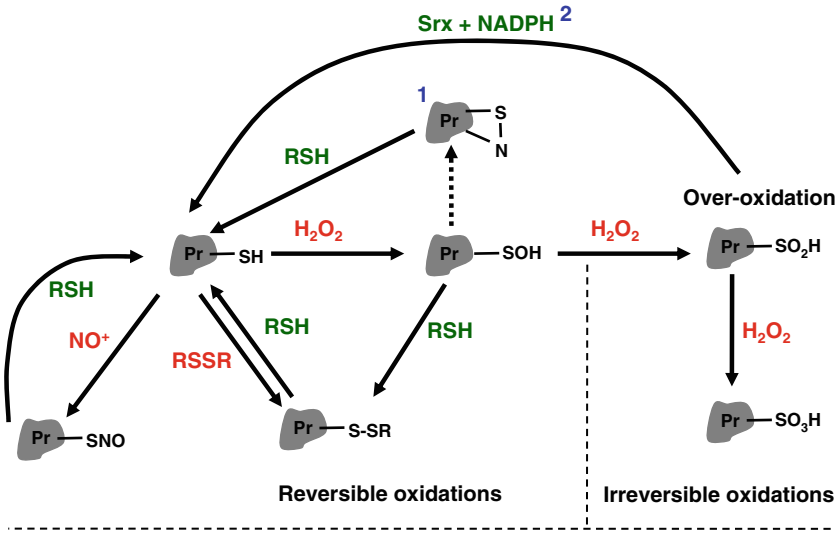

Fig. 1 Scheme of protein thiol oxidation and regeneration reactions. The scheme shows oxidation from protein thiol (Pr-SH) to S-nitrosothiol (–SNO), disulfide (–S-SR), sulfenic acid (–SOH), sulfenyl amide (–S–N–, labelled [1]), sulfinic acid ($–SO_2H$), and sulfonic acid ($–SO_3H$). The sulfiredoxin reaction is labelled [2]. At physiological pH the acids would normally be in the deprotonated form (sulfenate, sulfinate, sulfonate)

reaction is S-nitrosoglutathione (GSNO), from which GSH is recovered enzymatically by the action of GSNO reductase, with concomitant production of ammonia (Liu et al. 2001). The most common outcome of oxidation to sulfenate, mixed disulfide or S-nitrosothiol is reversible inactivation of the protein, as cysteines with a sufficiently low p*K*a to be susceptible to attack by H_2O_2 tend to be active site residues that are required for catalysis. Most proteins are also susceptible to overoxidation, i.e., further oxidation of the sulfenate to sulfinate or sulfonate forms (Rhee et al. 2005), and except in the case of some peroxiredoxins, this is an irreversible and damaging modification.

A classic example of this pattern of redox regulation is the protein tyrosine phosphatase PTP1B. PTPs contain an essential catalytic cysteine with a p*K*a of 4.7–5.4 within the signature motif [I/V]HCXXGXXR[S/T] (Peters et al. 1998). The cysteine is responsible for nucleophilic attack on the phosphate of the substrate to form a phosphothioester, and needs to be in the thiolate form to achieve this, so oxidation of the cysteine inactivates the enzyme. Elucidation of the role of cysteine oxidation in regulation PTP1B signalling dates back to 1998 (Lee et al. 1998), when it was shown using ^{3}H-iodoacetate labelling of cysteine and Edman degradation of PTP1B peptides that the active site cys^{215} was the major site of oxidation by hydrogen peroxide in recombinant PTP1B, and that EGF stimulation of A431 cells induced loss of iodoacetate labelling of PTP1B that correlated with loss of activity. Although PTP1B contains six cysteine residues, intramolecular disulfides are not thought to be formed, and only Cys^{215} is oxidized by hydrogen peroxide, even up to high 10 mM H_2O_2 (Lee et al. 1998; Lou et al. 2008). Further biochemical studies with purified PTP1B supported the suggestion by Lee et al. that the Cys^{215} was oxidized to a sulfenic acid intermediate, which could be re-activated by glutathione and other thiols, and it was also shown that glutathione disulfide could transiently inactivate the enzyme (Denu and Tanner 1998). Interestingly, in PTP1B, the sulfenate can also be converted to 5-membered cyclic sulfenyl amide intermediate by reaction with backbone nitrogen of Ser^{216} (Salmeen and Barford 2005; van Montfort et al. 2003). The sulfenyl amide can be reduced to the thiol by glutathione, and is thought to be protective by preventing oxidation of the cysteine to sulfinic or sulfonic acids, although as yet the formation of this intermediate has only been shown in oxidation in vitro.

There have been some studies of PTP1B regulation by reactive nitrogen species, demonstrating that S-nitrosylation also causes inactivation. Exposure of Jurkat or A431 cells to NO donors resulted in oxidation of PTP1B with concomitant loss of phosphatase activity, although it was concluded that the modification was more likely to be disulfide formation rather than S-nitrosothiol, based on the observation that the oxidation could be reversed by dithiothreitol but not by ascorbate (Li and Whorton 2003). However, more recent studies using mass spectrometry have demonstrated the formation of Cys^{215} SNO (Chen et al. 2007), while other PTP1B thiols remained unaffected. Treatment with either S-nitrosothiol reagents or NO radical donors did not result in any detectable irreversible oxidations of Cys^{215} (i.e., $–SO_2H$ or $–SO_3H$), and furthermore it was found that SNO formation protected PTP1B from over-oxidation to these forms induced by hydrogen peroxide (Chen et al. 2008).

The most common outcome of cysteine oxidation in an enzyme is inactivation, but a few cases are known where the oxidation results in gain of activity. This tends to involve changes in conformation leading to dissociation of inhibitory factors, then allowing gain of catalytic function in the case of an enzyme, or migration to the nucleus and

DNA binding in the case of a transcription factor. For example, the MAP3 kinase apoptosis signal-regulating kinase 1 (ASK1; MAPKKK5) exists as an inactive complex with thioredoxin, which can be dissociated by oxidative stress allowing activation of ASK1, which in turns promotes apoptosis via the p38 or JNK pathways. An early model of this regulation suggested that dissociation of Trx involved the oxidation of two cysteines (Cys^{32} and Cys^{35}) in the redox site of the Trx, as both intramolecular disulfide formation and S-nitrosylation of these cysteines had been observed in this context and cause activation of ASK1 (Matsukawa et al. 2004; Park et al. 2004). Subsequent studies refined the model to include oxidation of ASK1 cysteines resulting in intermolecular disulfide bonded multimers, and a more dynamic role for Trx more in line with its canonical role as a protein disulfide reductase (Nadeau et al. 2007). Another example is the transcription Nrf-2, which binds to promoters containing the electrophile-response element and is important in Phase II responses, is sequestered in the cytosol by an adaptor protein called Keap1 (Kelch-like ECH-associated protein). Keap1 mediates interaction with CUL-3-dependent E3-ubiquitin ligase, which ubiquitinylates Nrf2 leading to its degradation (Sekhar et al. 2009). Oxidation of two cysteine residues in Keap1 to an intramolecular, intersubunit, or mixed disulfide; this results in release of Nrf2 and its translocation to the nucleus. The human Keap1 contains 27 cysteines, many of which can be oxidized, but those currently thought to be most relevant to disruption of the Cul–E3 complex and Nrf2 release are Cys^{151}, Cys^{273} and Cys^{288} (Holland et al. 2008; Sekhar et al. 2009). It has also been suggested that Zn^{2+} binding to cysteines modulates their reactivity (lowering their p*K*a) and that release of Zn^{2+} on oxidation to disulfides is required for conformational change and Nrf-2 release (Dinkova-Kostova et al. 2002). A similar mechanism has also been suggested for the activation of heat shock protein 33 (Hsp33), which has 4 thiolates that bind Zn^{2+} and undergoes dimerization on oxidation to disulfides, and matrix metalloproteinases (MMPs) containing an active site Cys–Zn centre, where the Zn–thiolate interaction must be disrupted for activity (Gu et al. 2002; Jakob et al. 2000; Jakob et al. 1999; Janssen-Heininger et al. 2008). However, in MMPs it has been reported that activation requires HOCl or $ONOO^-$, and weaker oxidants nitric oxide and H_2O_2 are ineffective (Fu et al. 2001; Okamoto et al. 2004).

One of the most important questions about redox signalling is its physiological significance. Many studies that demonstrated the oxidation of cysteine residues have been carried out in vitro with hydrogen peroxide or other oxidants, and in some cases high concentrations (>0.1 mM) of hydrogen peroxide have been required in order to achieve oxidation. However, there are an increasing number of reports of redox effects following receptor activation in intact cells, in particular relating to the MAPK and PTP signalling pathways, which clearly provide physiological relevance. In much of the early work, receptor stimulation by insulin, EGF or PDGF was found to increase cellular levels of ROS or RNS, and in parallel oxidation of various signalling proteins was detected by immunoprecipitation and labelling with unmodified thiols with iodoacetate; this offers an indirect approach to identification of the oxidative modification, and detection of specific intracellular oxidants is sometimes open to criticism (Winterbourn and Hampton 2008). More recently, the development of mass spectrometric methods, as described below, has enabled the identification of specific cysteine oxidations under physiological conditions. For example, sulfenate, sulfinate and sulfonate products of Cys^{215} oxidation in PTP1B have been identified in cancer cell lines that constitutively produce oxidants, using MALDI-MS and MS/MS techniques (Lou et al. 2008).

The role of methionine oxidation in signalling

While cysteine oxidations in signalling are becoming very well established, the understanding of potential signalling functions of methionine has lagged somewhat behind; nevertheless, support for their role is now increasing. Methionine residues are very readily oxidized and can be repaired by methionine sulfoxide reductases; consequently methionine oxidation has previously been considered to exist as a protective mechanism for proteins, and one that in some proteins has comparatively little adverse on their function (Stadtman et al. 2003). Evidence for an additional role of certain methionines as oxidation sensors in the redox regulation of enzyme activity is accumulating, with particular emphasis on calcium transport and signaling (Emes 2009).

There are several studies reporting the effects of methionine oxidation in calmodulin (CAM), a calcium regulatory protein that leads to differential activation of many proteins, including the transcription factors Nrf-2, NFAT and NFκB, and is central in control of energy metabolism as well apoptosis. Calmodulin contains nine methionines, but only oxidation of Met^{144} and Met^{145} appear to contribute to redox control (Bartlett et al. 2003; Bigelow and Squier 2005). Oxidation of these residues affects the structure of the helix causing uncoupling of the calmodulin domains, in turn altering their binding to target proteins. For example, native calmodulin binds and the inhibitory subunit of the plasma membrane Ca^{2+}–ATPase to release inhibition, whereas met oxidation results in non-productive binding and stabilizes the inhibited state (Gao et al. 1998; Yao et al. 1996). Voltage-gated K^+ channels have also been found to be affected by oxidation of methionine residues (Hoshi and Heinemann 2001).

Ca/Calmodulin-dependent protein kinase II (CaMKII) is activated by increases in intracellular calcium, via binding of Ca/CaM which relieves inhibition (in a similar manner to the plasma membrane Ca^{2+}–ATPase mentioned above). Oxidation of met^{281} or met^{282} switches the kinase to Ca-independent activation, which does not require phosphorylation of Tyr^{287}, and leads to angiotensin-induced apoptosis in mouse cardiomyocytes (Erickson et al. 2008). In contrast, methionine oxidation reduces the activity of calcineurin, a Ser/Thr protein phosphatase whose activity is also regulated by CaM and which is involved in signalling in response to stress, especially in T cells. Thus, the opposing effects of methionine oxidation on calcium-dependent kinase and phosphatase signalling pathways seem somewhat analogous to those of cysteine oxidation in MAP kinase and phosphatase pathways. Demonstration of the wider relevance of methionine oxidation in signalling is illustrated by the report that MetSO formation in nitrate reductase in *Arabidopsis* inhibits the phosphorylation of Ser/Thr in adjacent sites, in situations where Met is hydrophobic recognition element in the consensus sequence (Hardin et al. 2009), which moreover emphasizes the interaction between phosphorylation and oxidation as regulatory mechanisms.

In addition to a clear emerging role in calcium signalling, there is also some evidence that methionine oxidation to sulfoxides may have a signalling role in NFκB activation. The studies so far have concentrated on effects of the oxidant HOCl and its downstream products such as taurine chloramine (Tau-Cl). HOCl (or NaOCl) inhibits NFκB activation by the cytokine TNFα, thought to be due to formation of a sulfoxide at Met^{45} on the inhibitory subunit IκB (Mohri et al. 2002), and similar findings have been obtained with Tau-Cl (Midwinter et al. 2006). As a result, ubiquitinylation and degradation of IκB is prevented. As degradation requires phosphorylation of IκB and the Met^{45} is located close to the phosphorylation sites Ser^{32} and Ser36, it has been suggested that sulfoxide formation causes a conformational change in the protein that prevents kinase interaction (Midwinter et al. 2006; Ogino et al. 2005). Overall, support for the concept of the role of methionines in cell regulation is growing (Agbas and Moskovitz 2009; Emes 2009).

Overview of analysis of thiol oxidation

In order to be able to understand the role of amino acid oxidation in biological processes, it is clearly important to be able to analyze in detail changes in the redox state of specific proteins of interest, and also to be able to detect cysteine oxidation or modification more globally in order to identify proteins with changed redox status. One fundamental difficulty with the study of cysteine oxidation is that thiols themselves, and many of their reaction products, are not intrinsically stable; thiols oxidize spontaneously to disulfides or higher oxidation states under aerobic conditions, alkylated thiol residues are prone to oxidation, and even disulfides are susceptible to further oxidation (Hawkins et al. 2009). Consequently, careful handling of the samples, or rapid labeling or other chemical modification procedures that stabilize and fix the redox state, is essential to allow accurate analysis of their role in biological signaling. Much work has been done on individual, purified proteins, and this has been invaluable in unraveling the location of the redox-sensitive residues and the characteristics of their redox cycle, with mass spectrometry playing the most important role in these studies. However, to identify new proteins involved in redox signalling and gain a more comprehensive overview of the extent of this phenomenon in cellular metabolism, it is necessary to detect these modifications both in specific proteins and more globally in cell or tissue extracts following appropriate stimulation. To this end, modern proteomics methods, especially mass spectrometry-based proteomic technologies, often combined with 2-dimensional (2D) electrophoresis, have proved extremely valuable.

2D Gel based analysis for thiol oxidation

2D gel based methods offer a straightforward global analysis of the more abundant cellular proteins or the detailed analysis of specific proteins alone or within complex mixtures. Even though some important classes of protein such as hydrophobic membrane proteins are missing from gels, and the limited resolving power and dynamic range, this technique has still proved to be a valuable method for both targeted and global analysis (Hampton et al. 2006; Leichert and Jakob 2004; Riederer 2009). Protein species appear as separated spots on the gel dependent on their physicochemical properties, most commonly their isoelectric point (pI) and size. Their location can be determined using non-specific protein dyes such as Coomassie blue, but there are more effective targeted approaches that utilize labeling of the proteins with a residue or modification-specific reactive chromophore (most often fluorescent), for example a maleimide- or iodoacetamide-based reagent for cysteine thiols (Bruschi et al. 2009; Fu et al. 2008; Riederer et al. 2008). There are many ways this technique can be utilized to detect modified cysteines. In one approach the two samples to be compared are labeled with different coloured chromophores, and then the two samples are mixed and run on the same gel, thus removing problems caused by variation between gels. Differential colour scanning is then used to detect the proteins and to analyze differences in abundance; this

procedure is often referred to as DIGE (differential in gel electrophoresis).

For standard 2DE analysis samples are usually reduced and the free thiols blocked with iodoacetamide to optimize performance of the gel. In an experiment seeking to identify redox sensitive groups, blocking of free cysteine thiols with a thiol specific reagent, followed by mild reduction (dithiothreitol, triphenyl phosphine, mercaptoethanol, etc.) and modification with the chromophore will label disulfides or sulphenic acids, or labeling first and then reduction/blocking will label free thiols. A comparison between the levels of oxidized or reduced thiol can then be made by comparing two samples generated under different conditions; for example control versus stressed. Immunoblotting for specific species is a selective alternative if antibodies are available. Spots of interest can be excised from the gel and the proteins identified by mass spectrometry, and (with luck) the site and nature of the modification might even be determined if the modified peptide is seen in the mass spectrometric analysis. An example of the use of this technique is the initial observation and identification of the nature of the oxidation of the active site cysteine of peroxiredoxin 1 in mammalian cells (Wagner et al. 2002), and in the demonstration of the reversibility of this oxidation to the sulphinic acid using S^{35} labelled cells and immunoblotting with an anti-prx1 antibody (Woo et al. 2003). The nature of the oxidation in the study was also confirmed by immunoenrichment of the protein and intact protein mass spectrometry showing a 32 Da shift in mass corresponding to the addition of two oxygens. These studies also demonstrated an additional advantage of the 2D gel approach, as the sulphinic modification causes a change in the pI of the protein resulting in a shift towards the acidic end of the gel, giving a clear visual indication of the oxidation, and also allowing straightforward relative quantification of the oxidized and reduced species.

A related technique that has potential to identify proteins with intra- or inter-molecular disulfides directly is diagonal 2D electrophoresis. Here both dimensions are run as denaturing SDS gels: the first dimension under non-reducing conditions, followed by in situ reduction of the proteins in the gel, and the second dimension under reducing conditions (Brennan et al. 2004; Cumming et al. 2004). Proteins appearing off the diagonal would be expected to have contained disulfides that altered their apparent molecular mass, for instance through intra-protein disulfides or conjugation with other thiols such as glutathione. While some signaling proteins were identified as being modified using this method, the functional significance of these modifications has not yet been demonstrated.

Mass spectrometric methods for disulfides and higher oxidation state sulfur

An alternative to the gel based approaches, and one that is receiving increasing attention, is the use of liquid chromatography coupled to mass spectrometry for the detection and analysis of oxidative protein modifications. There have been many reports of the direct observation of cysteine oxidation products either in vitro from the oxidation of proteins or peptides, or from in vivo sources using a range of mass spectrometric techniques. The methodology for the study of proteins is generally the same regardless of the source. The protein sample is initially digested with a specific protease, usually trypsin which cleaves at most lysine or arginine residues, although orthogonal digestions with alternative proteases such AspN, which cleaves at acidic residues and can increase the coverage of the protein sequence, are gaining popularity. The resultant peptides are either analysed directly by MALDI or ESI MS and MS/MS for relatively simple samples, such as purified proteins or model peptides, or are separated by liquid chromatography before analysis for more complex samples. Global analysis of the extent of oxidation of a protein can be achieved using mass spectrometry to measure the intact mass of a protein, which can usually be achieved to an accuracy of 1 in 10,000 or better with electrospray ionization, and hence can detect the addition of a single oxygen. This is generally limited to lower molecular mass proteins (<50 kDa) and does not identify the actual site of modification or the specific nature of the modification. It is, however, becoming increasingly feasible to use fragmentation of intact proteins with electron capture or electron transfer dissociation (ECD, ETD), in a "top–down" approach on high resolution mass spectrometers, to localize the sites of post-translational modifications, including oxidations (Pesavento et al. 2007).

Using mass spectrometric approaches a range of sulfur oxidation states can be analyzed directly. Oxidation to the sulfenic, sulfinic and sulfonic acids introduce one, two or three oxygens, increasing the mass of the oxidized peptide or protein by 16, 32 or 48 Da, respectively. Using these mass differences, sulphenic and sulphonic acid products have been identified in HOCl-treated group 4A Phospholipase A_2 (iPL$A_2\beta$) following trypsin digestion and LC–MS/MS analysis, and in this study MS was also able to identify the formation of an intermolecular disulfide leading to dimeric species and oxidation of a number of tryptophan residues with a mass change of 16 Da (Song et al. 2006). Sulfenic or sulfinic acids have also been observed by MS following the hydrogen peroxide oxidation of Cdc25, in both the intact mass of the protein and following tryptic digestion and LC–MS/MS analysis (Sohn and Rudolph 2003), and sulfenic, sulfinic and sulfonic acids

have been monitored in HOCl treated GroEL using a similar strategy (Khor et al. 2004). In this study, the protein was trypsin digested, and location of the susceptible residues was mapped by MS/MS fragmentation of the peptides. In the MS/MS fragmentation of modified peptides the change in mass is also visible and can be further localized to individual residues, identifying the modified residue. Furthermore, during the collisional activation characteristic neutral losses can be observed: these are loss of 50 Da ($-H_2SO$) from sulfenic acid-modified peptides (Shetty et al. 2007), 66 Da ($-H_2SO_2$) from sulfonic acid-modified peptides, which are most susceptible to cleavage (Wang et al. 2004), and 82 Da ($-H_2SO_3$) from sulfonic acid containing peptides (Summerfield et al. 1997). All of these neutral losses result in the formation of a dehydroalanine residue.

Similar approaches have been used to detect methionine sulfoxide formation. The formation of methionine sulfoxide can be detected in peptides as a mass increase of 16 Da, while overoxidation to methionine sulfone results in a mass increase of 32 Da (Xu and Chance 2005). It is well known that MetSO-containing peptides show a characteristic neutral loss of 64 Da (methanesulfenic acid, CH_3SOH) during collision-induced dissociation (CID) (Guan et al. 2003; Jiang et al. 1996), but the combination of CID with electron capture dissociation (ECD) using a FTICR mass spectrometer has proved particularly powerful for identifying methionine sulfoxide containing peptides, as ECD provides extensive peptide backbone fragmentation without loss of the labile side chain, which helps to locate the position of the oxidised methionine (Guan et al. 2003). Galeva et al. have monitored methionine oxidation in intact calmodulin under high energy CID conditions; this generated a series of fragments from residues 1 to 15 of the C-terminus containing the methionines of interest (Met^{144} and Met^{145}) (Galeva et al. 2005), and it was possible to measure the oxidation state of the intact protein and quantify the formation of methionine sulfoxide in a single experiment. The occurrence of methionine sulfone under more severe oxidation conditions has been detected in DJ-1 peptide from human brain from cases with Parkinsons disease using MALDI–TOF–TOF to detect the increases in m/z in the peptides containing Met^{17} and Met^{133} (Choi et al. 2006).

An indirect but commonly used approach to determining oxidation to labile sulfenic acids following cysteine oxidation is by trapping this as the more stable dimedone adduct, which can then be identified by mass spectrometry or, if appropriately derivatised, used as an affinity purification tag. The sulfenic acid modification of the single cysteine human albumin has been measured in this way by reaction with dimedone and analysis of the peptide containing Cys^{34}, which showed an increase in mass of 138 Da, consistent with the addition of dimedone, and comparison of the fragmentation of the unoxidised carbamidomethylated peptide with dimedone trapped oxidized peptide confirmed the oxidation on cysteine (Turell et al. 2009). A sulfenic acid in peroxide-treated tetrachlorohydroquinone dehalogenase was trapped as a stable dimedone thioether adduct in a similar way, and the site of oxidation identified as Cys^{13} using electrospray mass spectrometry (Willett and Copley 1996). The sulfinic acid of the same residue was observed directly by ESI/MS/MS in the overoxidised form of the terminal peptide from the protein both by mass increase and MS/MS sequencing. There have been a number of recent developments including the use of in vivo reagents with fluorescent tags which allow the modified proteins to be visualized on a gel or affinity tags which allow both enrichment and visualization (with a labeled streptavidin or antibody system) (Charles et al. 2007; Leonard et al. 2009; Poole and Nelson 2008). Reactive tags have also been developed, including azide analogues such as DAz-1N-(3-azidopropyl)-3,5-dioxocyclohexanecarboxamide, which after labeling of the sulfenate in vivo can then be specifically conjugated to a biotin affinity tag for analysis (Reddie et al. 2008) and other reactive groups, and antibodies with high specificity for the dimedone adduct have been produced (Seo and Carroll 2009). The dimedone method can been used to detect global changes in sulfenic acid modification in complex biological process; for example, the need for reversible sulfenic acid formation in the activation of T cells (Michalek et al. 2007).

There have been numerous other reports of the direct use of mass spectrometry to detect oxidative thiol modifications. The oxidation in vivo of Cys^{51} of peroxiredoxin II (PrxII) to its sulfinic and sulfonic acids was identified following AspN digestion of spots cut from 2D gels and negative ion MALDI–ToF mass spectrometry or ESI/MS/MS (Wagner et al. 2002), and these same modifications were also demonstrated for the equivalent residues in PrxI and PrxIII. AspN, which usually cleaves at the acidic residues asp and glu, was also shown to cleave the protein at cysteine that had been oxidized to the sulfonic acid. A more extensive mass spectrometric study of peroxiredoxin I (Prx I)-dependent NADPH oxidation was able to identify the sulfinic, sulfenic and sulphonic acids as cysteine oxidation products during turnover (Cho et al. 2004). Measurement of the intact mass of the protein showed a mass change equivalent to the addition of two or three oxygens were seen, and tryptic digestion followed by MALDI–ToF mass spectrometry was able to show that this was due to the oxidation of a single cysteine residue to the corresponding sufenic or sulfonic acid. This was further confirmed by ESI/MS/MS sequencing of the oxidized peptide, which demonstrated that Cys^{51} had been oxidized to the sulfenic acid. The retro-reduction of the inactive sulfinic

acid intermediate to the sulfenic acid by sulfiredoxin was monitored using rapid quench, ^{18}O labeling and mass spectrometry of the intact species, and from this it was possible to postulate a mechanism for the reaction via a thiosulfinate intermediate formed between the two proteins (Jonsson et al. 2008) The identification of novel oxidation products has also been facilitated by mass spectrometry. The formation of a sulphenyl-amide intermediate in the redox cycle of PTP1B was demonstrated by mass spectrometry using MS^2 and MS^3 studies, and the study was able to show that this was not an artifact (Shetty and Neubert 2009). A comprehensive analysis of nitroxyl (HNO) modification of cysteine-containing tryptic peptides by both collisionally induced and electron capture dissociation, and MS^3 studies demonstrated the initial formation of mainly the sulfonamide, which gave rise to a diagnostic 65 Da neutral loss (loss of $HSONH_2$) that could be used as a general method for identification of this modification, but the conversion of this to the sulfinic acid over time which gave rise to the diagnostic 66 Da neutral loss (Hoffman et al. 2009), corresponding to HSO_2H loss as mentioned previously.

Quantitative mapping of thiol–disulfide redox cycles, or other cysteine oxidation, can be achieved by mass spectrometry using mass tags in an analogous way to the fluorescent labels used in gel based analysis, but in this case the cysteine directed reagent has a "heavy" form produced by isotope labeling (usually ^{13}C or ^{2}H) and a "light" form containing the common isotopes (e.g. ^{12}C and ^{1}H). Samples to be compared are labeled with the light or heavy form, respectively, mixed together, proteolytically digested and analysed by LC–MS (Fig. 2). The equivalent peptides from the two samples elute from the chromatography at the same time, but can be distinguished in the mass spectrometer by the different masses, and hence quantified relative to each other. One of the most common reagents is the cICAT reagent from Applied Biosystems, which was initially developed as a cysteine-targeting mass tag for global quantitative proteomics (Hansen et al. 2003). The first extensive example identified changes in the thiol redox proteome in *E. coli* subjected to hydrogen peroxide or NaOCl stress (Leichert et al. 2008) using Tris(2-carboxyethyl)phosphine (TCEP) as the reductant. The authors refer to this technique as OxICAT, and noted that for the approximately 120 peptides they observed in each run, about 10% had only one tag, suggesting they were fully oxidized in vivo, but the rest showed variable modification. This method has been used to identify thioredoxin disulfide reductase targets in barley (Hagglund et al. 2008) where the extent of Trx-mediated reduction could be quantified for 104 out of 199 peptides identified as carrying the ICAT tag. The same approach has been used to study the effect of GSSG and peroxynitrite on p21ras GTPase, a putative signalling target for reactive oxygen and nitrogen species, by measuring quantitatively reactive free thiol groups (Sethuraman et al. 2007), and demonstrated that on peroxynitrite treatment cys^{80} labelling was unaffected but cys^{118} labelling decreased by 50%. Similar studies on human protein disulfide isomerase using this approach were able to differentiate different redox behaviours of cysteine pairs in the protein (Kozarova et al. 2007). A combination of fluorescence band mass tag based labeling has been applied to H_2O_2 treated heart tissue demonstrating that the 2 approaches are complementary, with 13 unique changes being identified with DiGE (fluorescence), 37 with ICAT, and only 13 in common; the identified proteins were mainly metabolic (Fu et al. 2008). A related quantification method has been developed by Landar et al., utilizing the comparison between an internal standard biotinylated on lysine and iodoacetamidobiotin tagging of free thiols (Landar et al. 2006).

Mass spectrometric methods for S-Nitrosothiols (SNOs)

The most commonly utilized method for identifying cysteine S-nitrosylation products (SNOs) is the biotin switch method, first described by Jaffrey et al. (Jaffrey and Snyder 2001), which relies on the reactivity of the free thiol in the same way as cICAT labeling (Fig. 2). Free thiol groups are first blocked, often using the mild blocking reagent methylmethanethiosulfonate (MMTS) which does not react with modified cysteine, followed by mild reduction of the nitrosylated thiols to the free thiol (commonly using ascorbate) and alkylation with a thiol-specific biotinylating agent, such as biotin–HPDP (N-(6-(biotinamido)hexyl]-3′-(2′-pyridyldithio) propionamide)) (Stuchbury et al. 1975). This method has been used to identify the S-nitrosyolation of a range of signalling proteins including caspase-3 (Mannick et al. 2001), NMDA receptor (Jaffrey et al. 2001), EF2 (Dall'Agnol et al. 2006), 14-3-3 s and annexins (Greco et al. 2006), NFκB (Miersch and Mutus 2005) and protein tyrosine phospatases (Barrett et al. 2005); a good, comprehensive review of this area is given by Lopez-Sanchez et al. (2009). Early studies on eNOS, the endothelial form of nitric oxide synthase, also utilized these methods. eNOS is regulated by a complex set of post translational modifications, and using a combination of classical biochemical methods and gel and mass spectrometry approaches it was possible to show that eNOS is S-nitrosylated in resting bovine aortic smooth muscle cells, but that receptor activation (VEGF) results in a rapid reduction in nitrosylation, which is dependent on other post-translational modifications including acylation and possibly changes in phosphorylation at Ser^{1197}, as well as membrane localisation (Erwin et al. 2006). The method

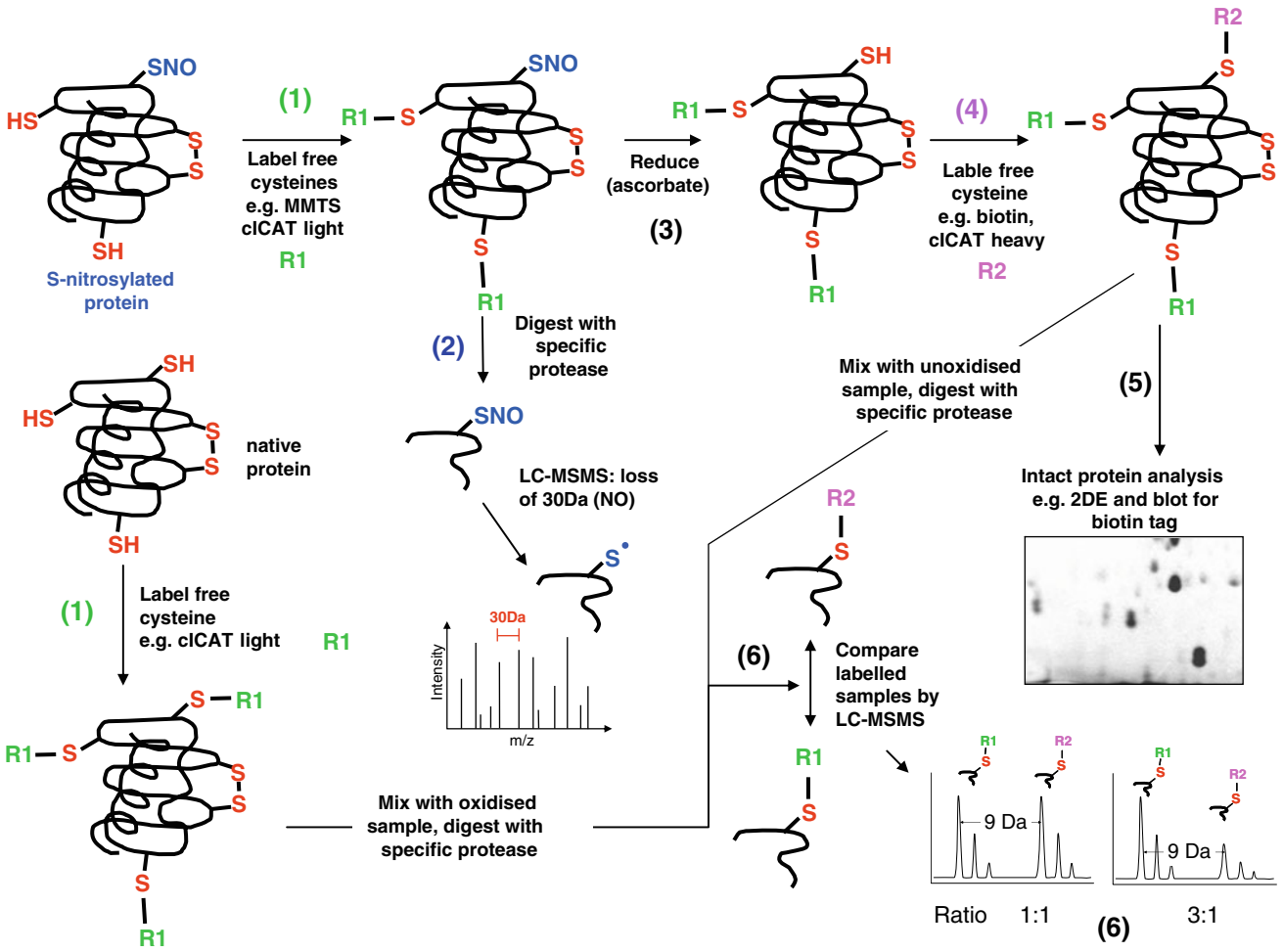

Fig. 2 Mass spectrometric approaches to detection and relative quantification of S-nitrosothiol formation. (1) Thiols in the native or oxidized protein are first blocked using, for example, methyl-methanethiosulfonate (MMTS) for direct analysis, or an ICAT label (e.g. cICAT light) to label free cysteines (shown as –**R1**). (2) S-nitrosothiols (–SNOs) can be detected directly by proteolytic digestion and MS, looking for the specific loss of NO. (3) SNOs can be selectively reduced, for example using ascorbate, which does not reduce disulfides. (4) the resulting thiol is labeled with a different tag (e.g. biotin or cICAT heavy, shown as –**R2**). (5) A biotin tag allows enrichment of the intact protein for analysis or visualization by western blotting of a 2DE gel, or affinity enrichment of the tagged peptides. (6) Alternatively, ICAT labelled native and oxidized samples can be mixed, proteolytically digested and analysed by LC-MSMS. (7) The amount of SNO formation can then be determined from the ratio of light to heavy ICAT tag, visualized by a difference in mass of 9 amu in the MS spectrum

appears to be sensitive enough to allow the isolation and identification of proteins endogenously S-nitrosylated from cells under conditions of basal SNO production (Mannick et al. 2001), and modification using a solid phase capture of the thiol, which appears to be better for high molecular mass proteins, and iTRAQ labelling (isobaric tag for relative and absolute quantitation: an alternative to cICAT that labels free amines of lysine and the N-terminus of peptides from the tryptic digestion) allowed a broad characterization of the endothelial cell SNO proteome in SNAP treated endothelial cells (Forrester et al. 2009). 89 proteins were detected as nitrosylated, and 29 of these could be identified. Hao et al. developed the related SNOSID method which introduced a tryptic digestion before the affinity isolation of the modified peptides and sequence determination by mass spectrometry (Hao et al. 2006). The S-nitrosylation sites in PKBα/Akt1 were determined using a similar approach (Lu et al. 2005), except that labelling of S-nitrosylated cysteines was performed using iodoacetic acid ($^{12}C/^{13}C$ ratio 50%) after ascorbate reduction but before disulfide reduction and capping with ^{12}C iodoacetamide. The PKBα/Akt1 was enriched by immunoprecipitation and the protein purified by 2D gel. Scanning for the immonium ion of cysteine in conjunction with the 50% ^{13}C labelling identified the modified peptides with reduced the false positive rate, and MS/MS was used to obtain sequence information. In some cases it has been possible to use specific mass changes for the direct identification of S-nitrosylation, although most of these studies have been performed in vitro. S-nitrosylation sites were identified by

the mass addition of 29 Da to the thiol on nitrosylation of ^{p21}Ras and synthetic peptides in vitro (Lander et al. 1997), and also in a synthetic peptide containing Cys^{62} (corresponding to the modified residue in vivo) from the NFκB p50 subunit treated with the NO generating systems sodium nitroprusside (SNP) or SNAP (Matthews et al. 1996). Site specific S-nitrosylation of Cys^{215} of PTP1B (as well as some other S-nitrosylation sites) was determined by Chen et al. using a number of MS approaches (Chen et al. 2008). Tryptic digestion of immunoenriched protein and MS showed an addition of 29 Da corresponding to the formation of a SNO, and MS/MS showed a neutral loss of 30 which corresponds to the loss of NO to give the cysteinyl radical. This neutral loss has potential for use as a selective method for detecting nitrosylation. In the same study, cICAT labeling of 1 and 0.01 mM SNAP modified protein and MALDI MS and MS/MS analysis (as well as X-ray studies) demonstrated no irreversibly oxidized forms and showed that in vivo S-nitrosylation protects against irreversible oxidation, and that the active site residues is the most susceptible to modification. However, direct analysis of nitosylated peptides requires careful optimization of the conditions (Wang et al. 2008).

oxPTMs at non-sulfur containing residues

Perhaps the best characterized role of oxidation of non-sulfur containing residues in signalling is the hypoxia-inducible factor (HIF) pathway (Kaelin and Ratcliffe 2008) that is used to sense normoxia and hypoxia in cells. Under normoxic conditions a set of proline hydroxylases (PHD 1–3) and an asparagine hydroxylase (FIH) (Lando et al. 2002; Mahon et al. 2001) are used to sense molecular oxygen levels. Proline hydroxylases use molecular oxygen, Fe(II), ascorbate and 2-oxoglutarate to oxidize two specific proline residues in the α-subunit of the heterodimric transcription factor hypoxia inducible factor (HIF) to 4-hydroxyproline, which targets this subunit for destruction via ubiquitinylation by the von Hippel–Lindau ubiquitin ligase, resulting in deactivation of the transcription factor (Fig. 3). Under hypoxic conditions, where molecular oxygen levels are reduced, proline hydroxylation is decreased and functional HIF dimer is formed resulting in transcription of a set of hypoxia induced genes. This process is fine tuned by FIH which also uses molecular oxygen, Fe(II), ascorbate and 2-oxoglutarate to hydroxylate a conserved asparigine in the α-subunit which prevents recruitment of coactivators and hence gene transcription (Kaelin and Ratcliffe 2008; Schofield and Ratcliffe 2004). The HIFs and FIH respond to different oxygen levels, giving fine tuning of the response, and are also regulated by other factors including reactive oxygen species, nitric oxide and metabolic status (Kaelin and Ratcliffe 2008). The hydroxylation of asparagines by FIH has now been demonstrated for a number of similar ankyrin repeats, including in other proteins involved in signalling (Notch 1–3, p105 and IκBα), and could be a more widespread regulatory mechanism (Cockman et al. 2009).

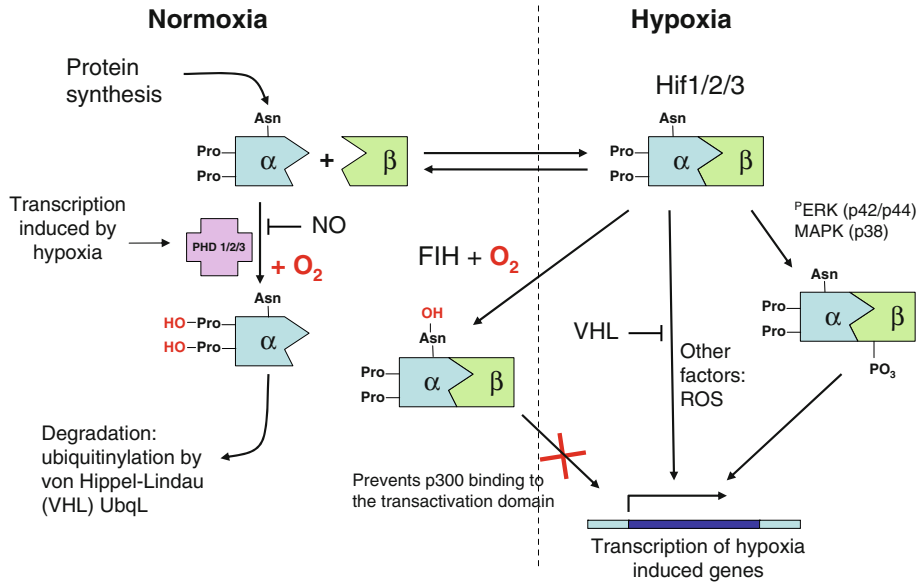

Fig. 3 Schematic view of the role of HIF and FIH in the control of hypoxia induced gene transcription. Under normoxia two prolines in the α-subunit of HIF are hydroxylated by PHD 1/2/3 utilizing molecular oxygen, which targets it for destruction via VHL-mediated ubiquitinylation and the 26S proteasome. Under hypoxia the α- and β-subunits combine to form the active transcription factor and gene transcription is turned on. Phosphorylation of the β-subunit is also required for activation. Fine control under a range of oxygen concentration is mediated by hydroxylation of an asparagine in the α-subunit by FIH, again utilizing molecular oxygen, which inhibits gene transcription. ROS are known to activate transcription although the mechanism is not yet established, and VHL inhibits transcription

While redox signaling involving sulfur oxidation and proline or asparagine hydroxylation has been extensively studied, and is well understood in a number of proteins, the role of other oxPTMs in redox signaling is much less clear. A number of other residues are susceptible to oxidative or nitrosative attack, especially tryptophan, histidine, tyrosine and proline. Tyrosine nitration has previously mostly been regarded as a stable PTM and indicator of pathological damage. However, there have been recent suggestions that it could contribute to redox regulation. It can interfere with tyrosine phosphorylation as the pKa of the phenolic group is lower in nitrotyrosine than tyrosine, thus modulating signalling by protein tyrosine kinases or phosphatases (Radi 2004). There is limited evidence that the formation of nitrotyrosine can alter protein activities, either as loss of function such as with Mn superoxide dismutase (Guo et al. 2003), or involving gain of function, which has been observed with protein kinase Cε and cytochrome *c* (Balafanova et al. 2002; Cassina et al. 2000). However, for tyrosine nitration to qualify fully as a redox signalling mechanism, the modification must be reversible within a suitable timeframe. Evidence for an enzymatic denitration system that is independent of proteolysis has been found in a variety of tissues, but considerable further work is required to demonstrate a physiological role in signalling (Abello et al. 2009; Rubbo and Radi 2008). In order to address this question, sensitive detection techniques that can identify the specific protein tyrosines nitrated in cellular experiments are required. Additionally, these methods will assist in the identification of protein oxidation biomarkers for redox-related diseases.

Direct detection of oxPTMS at non-sulfur residues: identifying diagnostic ions

Of the non-sulfur-containing residues that undergo oxPTMs, much attention has focused on the detection of nitrotyrosine, largely owing to its perceived value as a marker for nitrative damage in vivo. Proteins containing nitrotyrosine can be detected following 2D electrophoresis by western blotting with anti-nitrotyrosine antibodies, and by parallel mass spectrometric analysis of protein digests from the corresponding gel spot. As with cysteine modifications described above, identification of the protein and site of modification require the application of MS/MS procedures to sequence the peptides and confirm the presence of nitrotyrosine. The detection of histidine or tryptophan oxidation, or alternative oxidation products of tyrosine such as 3-hydroxy- or 3-chlorotyrosine, are also of interest but they rely entirely on mass spectrometric approaches, as specific antibodies for these modifications are not available. Some studies investigating oxidation of tyrosine or tryptophan residues have been carried out in vitro using purified proteins, and have involved peptide sequencing of tryptic digests using LC–MS/MS or MALDI–MS/MS to determine the site of modification. The products of tryptophan oxidation hydroxytryptophan, kynurenine and N-formylkynurenine have been observed in tryptic peptides of α-crystallin by this approach (Finley et al. 1998). 3-nitrotyrosine was observed in $ONOO^-$-oxidized GroEL by the observation of an increase of 45 amu in the nitrated compared to the non-nitrated peptide in collision-induced dissociation MS^2 spectra (Khor et al. 2004). Similarly, 3-chlorotyrosine formation was detected in Apo-AI protein from human HDL following treatment with MPO-H_2O_2–Cl^- by LC–ESI/MS/MS of tryptic peptides; tyrosine chlorination was identified by a mass increase of 34 amu and was found to occur preferentially at Tyr 192, although some chlorotyrosine formation was also observed from four other tyrosines (Bergt et al. 2004).

The strategy employed in these studies is convenient for the investigation of purified proteins, but cannot readily be applied to complex biological mixtures where the oxidation target(s) is unknown, as their identification would necessitate the MS^2 sequencing of all the proteins in the mixture. For complex samples, selective approaches such as precursor ion scanning or neutral loss are more desirable. Precursor ion scanning involves peptides containing the modification of interest fragmenting to give a diagnostic MS^2 daughter ion whose detection indicates the presence of the oxPTM in the parent ion, while neutral loss involves loss of a diagnostic mass between the parent and daughter ions that likewise signals presence of the oxPTM; both rely on the existence of a diagnostic transition for the oxPTM in question. Such "label-free" methods have been developed and used for monitoring phosphorylation, glycosylation, sumoylation and ubiquitinylation (Johnson and Gaskell 2006), and have more recently been applied to analysis of oxPTMs. In one study, angiotensin II and bovine serum albumin were nitrated in vitro with tetranitromethane, and the formation of nitrotyrosine was detected by precursor ion scanning for the nitrotyrosine immonium ion at *m/z* 181.06 (Petersson et al. 2001). The site of modification was identified by mass mapping of the peptide, which can also identify the source protein if necessary. This methodology has recently been investigated further for analysis of 3-chlorotyrosine, 3-nitrotyrosine, hydroxytyrosine and hydroxytryptophan, but it was found that the immonium ions (Y–Cl *m/z* 170.1; Y–NO_2 *m/z* 181.1; Y–OH *m/z* 152.1; W–OH *m/z* 175.1) formed by fragmentation in a standard MS^2 protocol were not sufficiently selective (Mouls et al. 2009). A significant false positive rate was encountered owing to the existence of isobaric ions at these masses in the MS^2 fragmentation, especially for *m/z* 170.1 and 181.1.

Consequently a novel MS^3 strategy was developed to obtain more specific diagnostic fragment ions, and was found to be successful for chlorotyrosine (MS^3 ions at *m/z* 125.1, 134.1, 153.1), hydroxytyrosine (MS^3 ions at *m/z* 107.1, 135.1), 2-hydroxy tryptophan (MS^3 ions at *m/z* 130.1, 158.1) and 5-hydroxytryptophan (MS^3 ions at *m/z* 146.1, 148.1 158.1) in BSA, a mixture of 9 proteins, or an *E. coli* lysate (Mouls et al. 2009). However, even with MS^3 fragmentation there was still a high false positive and a significant false negative rate for nitrotyrosine.

An alternative approach to detection of nitrotyrosine is to use specific tagging procedures to label the modified protein; this has additional advantages. Firstly, nitrotyrosine-containing proteins occur at low relative abundance in vivo, even in pathological samples, and the tagging groups can be used to capture and enrich these proteins. Secondly, labelling offers the opportunity to use isotope coding to determine the relative abundance of the nitrotyrosine modification. The nitro group, in contrast to thiols, is relatively unreactive, and must first be converted to an amine, a reaction that is usually achieved with dithionite. A very simple procedure involving nitrotyrosine conversion to amino tyrosine, followed by acetylation, has been reported for the site-specific identification of nitrotyrosine in BSA by LC/MS/MS (Ghesquiere et al. 2006), but offers limited advantages over the direct analysis of nitrotyrosine. Dansylchloride has been used by Amoresano et al. (2007) to label aminotyrosine; this allowed the nitrotyrosine-containing peptide to be detected by scanning for precursor ions of *m/z* 170, with further confirmation of the presence of the dansyl group using the characteristic transition *m/z* 234 to 170 in MS^3 fragmentation. This methodology was applied to nitrated BSA and also nitrated milk proteins, as a more complex mixture. CID was used to sequence peptide for identification in BSA and bovine milk protein extract. The main disadvantage was that the efficiency of dansylation was only 60%. The same group subsequently used the same protein models to develop a quantitative approach to nitrotyrosine detection, combining reduction to aminotyrosine with use of iTRAQ labelling reagents. Up to four samples can be labelled with the different iTRAQ reagents; the labels are released in MS^2 and detected at *m/z* 114/115/116/117, so they can be used for differential precursor ion scanning. As the peptide backbone fragments generated are the same for all samples, this improves the signal-to-noise ratios during the CID scan for protein identification (Chiappetta et al. 2009). Some combined labelling and capture strategies have also been described. Lee et al. describe the reduction of nitrotyrosine to aminotyrosine, and conversion to monopyridinylmethylamine in several steps (Lee et al. 2009). The labelled peptide was enriched by immobilized metal ion affinity chromatography with nitrilotriacetic acid Ni^{2+} magnetic beads, and the procedure was tested with nitrated angiotensin and BSA either individually or spiked into a tryptic digest of a Hela cell lysate.

Specific methods for the detection of 4-hydroxyproline and hydroxyasparagine are much less developed. An immonium ion scanning method for the detection of hydroxyproline has been described for the analysis of hydroxylated peptides from *Conus textile* (Steen and Mann 2002) which uses high mass accuracy to discriminate the hydroxyproline immonium ion (*m/z* 86.061) from other amino acids with the same nominal mass, e.g. leucine/isoleucine (*m/z* 86.097). However, this has not yet been applied to the analysis of PHD substrates. The standard methods for determining hydroxyproline formation in the PHD/HIF system is to incubate with synthetic biotinylated peptides containing the proline hydroxylation sites and then to recover these and analyze them by MALDI–TOF or electrospray mass spectrometry (Hewitson 2007). Detection of substrates for asparagine hydroxylation by FIH has been performed by immunoprecipitating the FIH and identifying associated proteins using proteomics approaches (Cockman et al. 2006, 2009), but no mass spectrometric methods for the direct detection of asparagine hydroxylation have been reported. Notch1-3, p105 and IκBα were identified using this approach.

All of these approaches have advantages and disadvantages; labelling methods offer selectivity and specific MS transitions that can aid detection, but involve extensive sample manipulation that can lead to artifacts and loss of sample, and may be avoided with the direct MS analysis of oxPTMs.

Conclusions

In summary, it can be seen that redox signaling is a well-established phenomenon that is fundamental in stress response pathways in cells, and additionally modulates a variety of other processes from metabolic pathways to the cell cycle. The best understood mechanisms involve oxidation of cysteine residues, but support is growing for the concept that nitration of tyrosine could contribute to regulation of signaling. Although considerable detail is available about the redox reactions affecting the activity of a few specific signalling proteins, there are still many gaps in our understanding of how redox signaling is integrated, the achievement of specificity, and the source and nature of the oxidants. The identification of precise oxPTMs involved in redox signaling processes that has been achieved for some proteins has depended largely on the enormous improvement in analytical techniques over the last few years. In particular, improved sensitivity and increased sophistication in mass spectrometry have enabled

the identification of redox sensitive proteins and mapping of the sites of oxidation therein. However, it is important to remember that global screening protocols tend to highlight changes in abundant proteins, which are not necessarily those of most functional relevance; this is most problematic in signalling studies, but may also impact on identification of disease specific biomarkers.

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