Abstract: In the cardiovascular system, changes in oxidative balance can affect many aspects of cellular physiology through redox-signaling. Depending on the magnitude, fluctuations in the cell's production of reactive oxygen and nitrogen species can regulate normal metabolic processes, activate protective mechanisms, or be cytotoxic. Reactive oxygen and nitrogen species can have many effects including the posttranslational modification of proteins at critical cysteine thiols. A subset can act as redox-switches, which elicit functional effects in response to changes in oxidative state. Although the general concepts of redox-signaling have been established, the identity and function of many regulatory switches remains unclear. Characterizing the effects of individual modifications is the key to understand how the cell interprets oxidative signals under physiological and pathological conditions. Here, we review the various cysteine oxidative posttranslational modifications and their ability to function as redox-switches that regulate the cell's response to oxidative stimuli. In addition, we discuss how these modifications have the potential to influence other posttranslational modifications' signaling pathways though cross-talk. Finally, we review the increasing number of tools being developed to identify and quantify the various cysteine oxidative posttranslational modifications and how this will advance our understanding of redox-regulation. (Circ Res. 2013;112:382-392.)

Key Words: cardiac protection ■ cysteine oxidative posttranslational modification ■ heart failure ■ protein ■ redox-switches
Unique Characteristics of Cys and its OX-PTMs

The side chain of a Cys residue contains a terminal thiol (–SH) functional group. The sulfur atom at the core of the thiol is electron rich and its d-orbitals allow for multiple oxidation states.4,5 The availability of different oxidation states permits the formation of a diverse range of OX-PTMs including S-nitrosylation (also called S-nitrosation; SNO), sulfhydration (SSH), S-glutathionylation (RS-SG), disulfide bonds (RS-SR'), sulfenylthionylation (SHT), and sulfenic acid (SOH). As shown in Figure 1, most Cys OX-PTMs are stimulated by diffusible small molecules and are reversible. They can be reduced to a free thiol (SH) by the antioxidant defense system or converted to other OX-PTMs depending on the cell’s redox-state. The formation of an individual OX-PTM can depend on many factors including the reactivity of the individual Cys residue, its surrounding environment, and the composition of the local redox-environment. A summary of the various OX-PTMs and the redox-chemistry associated with their formation is presented in the Table. Most Cys OX-PTMs are induced by ROS/RNS molecules that react with the free thiol on a Cys side chain.

S-Nitrosylation

ROS/RNS can react with a thiol group either by a single or double electron transfer resulting in the formation of an OX-PTM (Figure 1). Previous studies have mainly focused on reversible Cys OX-PTMs in cardiovascular biology. Within these studies, there is an emerging role for SNO in cardioprotection.33,34 Seminal papers by Murphy and colleagues35–38 have suggested that SNO modification is a central phenomenon for cardioprotection. Sun et al proposed that SNO modification of critical thiols during ischemic preconditioning (mild ischemic/oxidative stress) shields them from further oxidative (irreversible) damage during a longer or more severe ischemic insult ensuring that the cell can subsequently regain normal function quickly. This concept is supported by recent work from our group which demonstrated SNO of ATP synthase at Cys294 was increased in cardiac resynchronization therapy (CRT) in a canine model. In this study, there is competition among different OX-PTMs for the same Cys residue (C294) in ATP synthase.26 Excitingly, the actual OX-PTM occupying this Cys residue differed depending on the phenotype: control versus HF or when the HF animals were treated with CRT.36 This suggests that C294 in the ATP synthase α-subunit may function as a redox-switch.

To determine which Cys residues act as redox-switches requires the identification of residues sensitive to multiple OX-PTMs. We compiled the first database for identified SNO-modified proteins (and the modified site) from 4 different proteomic studies on cardiac tissue,10,35,37 endothelial cells,10 and smooth muscle cells28 (online Table 1). The SNO-modified proteins were found to be involved in a variety of cellular functions, including energy production. The 3 methodologies used to capture and identify SNO-modified proteins are discussed later in the review. As shown in Figure 2, there is little overlap in the proteins identified by the various methods although this may, in part, reflect differences in the sample fractionation and sample types. Only 15 proteins are found in common between 2 of the 3 methods. Of these, 6 are involved in mitochondria/metabolism, the remainder of the group comprises ion channel and several nuclear proteins. In total, 359 SNO-modified Cys were identified which corresponded to 258 nonredundant proteins. This represents 15% of 2525 total Cys residues present in these 258 modified proteins (Figure 2A). Of these, the majority of the proteins had only a single SNO-modified Cys (Figure 2B). This suggests that there is a high degree of specificity for the residues that are modified. However, previous attempts to determine a linear amino acid consensus sequence have been largely unsuccessful, suggesting that other physical characteristics play a role in reactivity. Another interesting aspect of these data sets was the opportunity to assess the subcellular localization of the SNO-modifiable proteins. These proteins were found to be disturbed across many cellular organelles including the mitochondria, nucleus, plasma membrane and cell junction, as well as the

<table>
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<tr>
<th>Non-standard Abbreviations and Acronyms</th>
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<tr>
<td>CRT</td>
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<td>Cys</td>
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<td>ER</td>
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<td>OX-PTM</td>
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<td>PTM</td>
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<td>ROS/RNS</td>
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<td>SNO</td>
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<tr>
<td>SOH</td>
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<tr>
<td>S2O3H</td>
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<td>RS-SG</td>
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During the last 2 decades, reactive oxygen species/reactive nitrogen species (ROS/RNS) have been found to function as important physiological regulators of intracellular signaling pathways.1,2 The specific effects of ROS/RNS are modulated, in part, through the covalent modification of specific cysteine (Cys) residues found within redox-sensitive proteins. Oxidative posttranslational modification (OX-PTM) of Cys residues is an important mechanism that regulates protein structure and ultimately function. The unique properties of a Cys side chain permit a variety of OX-PTMs, which potentially result in diverse regulatory effects.3 In this review, we focus on the unique aspects of Cys OX-PTMs that have the potential to regulate cell phenotype. We also discuss recent findings examining how each Cys residue can have different reactivity to the Cys OX-PTMs, provide examples of how OX-PTMs can modulate the function of different target proteins, and review a small subset of Cys residues that have been found to act as redox-sensors or switches that may compete for different OX-PTMs. The latter we speculate contributes to the coordination of the cellular response to oxidative stress. Finally, we discuss the increasing number of tools being developed to identify and quantify the various Cys OX-PTMs and how this will continue to advance our understanding of redox-regulation.
cytoskeleton (Figure 2C). This indicates a potential for SNO to have a broad effect on the cell. Based on this assumption, we hypothesized that this could be a possible mechanism for a coordinated cellular response to RNS.

**Sulfhydration**

To date, hydrogen sulfide (H$_2$S) has been proposed mostly to be a physiological vasorelaxant.$^{39,40}$ Transgenic mice lacking cystathionine γ-lyase, an enzyme involved in the synthesis of H$_2$S, were found to be hypertensive.$^{41}$ Despite its known biological effects, only a small number of target proteins have been identified. Recently, H$_2$S-mediated SSH has been suggested to be an important Cys modification, resulting in the formation of a persulfide bond (R-SSH). Some sulfhydrated proteins have been identified using liquid chromatography-tandem mass spectrometry,$^{21}$ although the mechanism for how these modified proteins experience the physiological effects of SSH is not yet clear. Recently, expanding roles for H$_2$S and its target protein were proposed by Krishnan et al.$^{42}$ They suggested that H$_2$S is the key aspect of the endoplasmic reticulum (ER) stress response. H$_2$S produced by cystathionine γ-lyase in response to ER stress sulfhydrates protein tyrosine phosphatase 1B (PTP1B) and decreases its activity. This results in the inhibition of the PTP1B-mediated dephosphorylation of protein kinase-like ER kinase (PERK), thereby facilitating PERK phosphorylation and its activation during the response to ER stress.

**S-Glutathionylation**

S-Glutathionylation (RS-SG) is the formation of a reversible RS-SG modification. It is formed by a reaction of GSSG (glutathione, oxidized form) or GSNO (S-nitrosoglutathione) with free thiol through either a single electron via thyl radicals (RS$^\bullet$) or dual electron pathways. An S-nitrosylated thiol is one of the possible intermediates for RS-SG and it can also be formed from sulfenylated thiols or sulfenylamide by reacting with GSH (Figure 1). Some proteins involved in cardiovascular disease, diabetes mellitus, lung, or neurodegenerative disease, have been found to be S-glutathionylated.$^{43}$ Studies for the physiological effects by RS-SG are still being elucidated and 1 such study about ATP synthase is discussed below.

**Disulfide Bonds**

Disulfide bonds (RS-SR$'$) are formed between the thyl radicals of 2 independent free thiols that are in close proximity, either within a protein or between proteins (termed an intramolecular disulfide bond, respectively). Disulfides can also be formed by reaction with sulfenic acid (Figure 1). In this case, the presence of high concentration of ROS converts SOH groups to thyl radicals (RS$^\bullet$), which react with other thiolates preferentially to form a disulfide bond.$^{5,44}$ Disulfide bonds are often involved in protein folding or complex formation and are assumed to induce a static protein conformation.$^{45}$ Recent work has suggested that some disulfide bonds are dynamic and can confer changes in protein structure and function (see Discussion below on ATP synthase).

**Sulfenylation and Irreversible Modifications**

Sulfenylation (SOH) is the lowest oxidation state induced by the ROS hydrogen peroxide (H$_2$O$_2$) and superoxide ($\bullet$ O$_2^-$). It has long been considered as deleterious oxidative damage but during the last 10 years SOH has been emerging as a critical intermediate in redox-signaling.$^5$ Sulfenic acid is reactive, unstable, and is believed to be short lived in the cell.$^{46}$ Because of its reactive nature, sulfenic acid can be converted to other Ox-PTMs, reversible or irreversible modifications depending on the local redox-environment (Figure 1). As a reversible modification, it can react with the abundant tripeptide glutathione (Glu-Cys-Gly, GSH), forming S-glutathionylated Cys (RS-SG) or react with a nearby available thiol to form a disulfide bond (RS-SR$'$). It has been suggested that once RS-SG (or RS-SR$'$) has been formed, these modifications could protect the host Cys from additional oxidative reactions.$^5$ This is feasible.
as both RS-SG (and RS-SR') are largely reversible by the cell's redox-defense mechanisms.

An alternative path for SOH is to undergo further oxidation directly to the irreversible Ox-PTMs, SO2H, and then SO3H, both having a higher oxidation state. Although there are examples where SO2H modification can be reversed enzymatically,47–49 these Ox-PTMs have been primarily associated with higher levels of ROS/RNS and are largely viewed as irreversible oxidative damage in the cell.

**Differential Reactivity: Not All Cys Residues Are Created Equally**

Not all Cys residues react equally to changes in local redox-state. This diversity in reactivity provides the basis for specificity in ROS/RNS-mediated signaling. As mentioned above, although a given protein may contain numerous Cys amino acids, only a minority of these have the availability and chemical properties to function as possible target sites for ROS/RNS. Redox-reactivity depends on both known and unknown factors, such as the local redox environment and the structural context of the Cys residues.

### Table. Chemistry and Methods of Cys Ox-PTMs

<table>
<thead>
<tr>
<th>Mediator</th>
<th>PTM</th>
<th>Reactions by Mediator (Two Electron Transfer)</th>
<th>Reactions by Thiyl Radical (Single Electron Transfer)</th>
<th>Methods for Detection</th>
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<tr>
<td>GSNO</td>
<td>S-Nitrosylation (SNO)</td>
<td>P-SH + RSNO → P-SNO + RSH</td>
<td>PS* + NO* → P-SNO</td>
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<tr>
<td>N2O3</td>
<td></td>
<td>N2O3 + RSH → P-SNO + H2O</td>
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<td>Biotin-HDPO26</td>
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<td>SNO-RAC7</td>
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<td>CysTMT10</td>
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<tr>
<td>H2O2</td>
<td>Sulfenylation (SOH)</td>
<td>P-SH + H2O2 → P-SOH + H2O</td>
<td>PS* + O2 → PSO0•</td>
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<td>PS0• + RSH → RS0• + P-SOH</td>
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<td>P-SO2H + H2O2 → P-SO2H + H2O</td>
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<td>Direct detection by MS18</td>
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<td>Direct detection by MS19,20</td>
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<tr>
<td>H2S</td>
<td>Sulfhydrylation (SSH)</td>
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<td>Biotin switch style15,22</td>
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<td>GSSG</td>
<td>S-Glutathionylation (RS-SG)</td>
<td>P-SH + GSSG → P-SSG + GSH</td>
<td>PS* + GS− → PSSG*</td>
<td>Biotinylated reagent12,23</td>
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<td>PSSG* + O2− → PSSG + O2−</td>
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<td>Thiol-Sepharose26,27</td>
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*Mechanism has not been demonstrated, yet.

![Figure 2. Overview of the S-nitrosylation (SNO)-modified cysteine (Cys) residues. A, The distribution of the 359 modified sites identified based on different experimental protocols is described in the text. B, Distribution of modified sites identified per protein. C, The subcellular localization of modified proteins as determined by annotation in the Uniprot database. CysTMT indicates cysteine tandem mass tag; and RAC, resin-assisted capture.](http://circres.ahajournals.org/)
It is known that the physical availability and pKₐ of a thiol can impact reactivity of a particular Cys residue. The average pKₐ of a thiol is around 8.6; however, this can vary from 3.5 to 10 depending on the molecular environment that surrounds the individual thiols.⁵⁰,⁵¹ Thiols act as a nucleophile and become more reactive in their thiolate form (Cys-S⁻); the lower a thiol’s pKₐ, the more likely it will be deprotonated and reactive. One factor that can lower the pKₐ is the composition of the neighboring amino acid residues. A thiolate anion is stabilized by positively charged or protonated amino acids but destabilized by negative charges. Witt et al.⁵² demonstrated that the oxidative sensitivity of Cys106 in DJ-1 (Parkinson disease protein 7), a protein which protects cells against oxidative stress, is determined by neighboring amino acids’ side chains. They found that the protonated glutamic acid (E18) reduced pKₐ of Cys106 from 8.3 to 5.4 by stabilizing the interaction with the thiolate. Despite these influences, no amino acid consensus sequence has been identified for predicting the reactivity of the neighboring amino acids’ side chains. They found that the protonated glutamic acid (E18) reduced pKₐ of Cys106 from 8.3 to 5.4 by stabilizing the interaction with the thiolate. Despite these influences, no amino acid consensus sequence has been identified for predicting which Cys residues may be targets for redox-regulation. This suggests that pKₐ, hydrophobicity and primary and secondary structure (including an acid/base pocket near the SNO-reactive Cys)⁵⁰ may contribute to different degrees toward an individual thiol’s reactivity⁵¹ rather than to a single unified consensus sequence.⁵⁰

The consequence of Cys having differences in their pKₐ is that proteins can vary in their reactivity to Ox-PTMs. Two recent proteomic studies have addressed this point. The first study used a general thiol-reactive iodoacetamide-based probe and identified a large number of Cys-modifiable proteins in 3 human cell lines.⁵⁴ The authors reported that general Cys reactivity predicts that the most highly reactive sites are likely involved in an active site and have greater regulatory potential. In the second study, our group used a novel reagent (cysteine tandem mass tag, described below) to determine the reactivity of Cys specifically for SNO-modifications.⁵⁰ Endothelial cell lysates exposed to increasing concentrations of NO-donor showed that most of the available Cys residues were not modified. However, the Cys residues that were susceptible to SNO modification showed a continuum of reactivity. Some Cys residues were very sensitive to SNO modification, whereas others were less sensitive. Endothelial cells in culture exposed to increasing oxidative stimuli resulted in the modification of more of the insensitive Cys residues. Based on these findings, it is tempting to speculate that differences in thiol-reactivity comprise a coordinated mechanism for the cell to gauge the magnitude of an oxidative stimulus and respond accordingly.

Redox-sensors and Redox-switches: ATP Synthase and α-Actin

Because Cys residues can have different reactivity to oxidation, it is reasonable to hypothesize that each Cys Ox-PTM have a unique reactivity curve. Thus, for a given ROS/RNS exposure there would be a selective group of Cys containing proteins that may be receptive to different Ox-PTMs. It is this subset of proteins, where various Cys Ox-PTMs compete for a single Cys residue, which could be reactive hubs and act as a cellular redox-sensor capable of monitoring and coordinating the cell’s redox-balance. The term redox-sensing is commonly used to describe global Cys-dependent control mechanisms, whereas redox-signaling refers to a specific signaling process in which a redox-element transmits an activation/inhibition signal.⁵⁵ As such, redox-sensors are defined as switches that control the cellular redox-homeostasis and redox-switches are biochemical components that control the redox-state of the reactive Cys in proteins. The concept of Cys redox-switches is emerging as a significant regulatory mechanism in the cardiovascular system and elsewhere.⁵⁶,⁵⁷ Modifications of this type have been found to affect cellular signaling, impacting both physiological and pathological pathways.⁵⁷ In addition, redox-compartmentalization can have a dramatic impact on redox-switches because different intracellular compartments have different redox characteristics, including protein composition, pH, reducing power, and sources of ROS/RNS generation.⁵⁸ The following sections review some selected examples of redox-switches involved in cardioprotection, HF, ischemia/reperfusion injury, and cardiac hypertrophy and specifically focuses on mitochondrial F₁Fₒ-ATP synthase and myofibril actin.

ATP Synthase

F₁Fₒ-ATP synthase (also known as complex V) is located on the mitochondrial inner membrane, chloroplast thylakoid membranes, and bacterial plasma membranes. Its role is to synthesize ATP from ADP and phosphate at the expense of the proton gradient generated by the electron transport chain across the mitochondria membrane.⁵⁹ The F₁Fₒ-ATP synthase complex is responsible for the majority of cellular ATP production, and as a result, is very tightly regulated.⁶⁰ ATP synthase consists of multiple highly conserved core subunits including α, β, and γ, which are located in the F₁ catalytic domain and other less conserved subunits which differ between plants and other life forms⁶¹-⁶３ (Figure 3A).

Redox-regulation of plant chloroplast ATP synthase is well documented.⁶⁴ It involves the formation and reduction of a disulfide bridge between 2 Cys residues located in the γ-subunit. Reduction of the disulfide bond shifts the threshold for ATP synthesis toward lower proton gradients making it easier for the mitochondria to produce ATP.⁶⁴ Interestingly, the chloroplast redox-sensitive γ-subunit region is not present in mammalian cells; however, mitochondrial F₁Fₒ-ATP synthase complex is still a hot spot for Ox-PTMs.

We have directly shown in an animal model of dysynchronous HF that the mitochondrial ATP synthase is regulated through the site-specific Ox-PTMs of some of its subunits.²⁶ During HF, the ATP synthase α-subunit forms disulfide bonds between Cys294 on neighboring α-subunits and to γ-subunits at Cys103. Furthermore, Cys294 in the α-subunit can also be S-glutathionylated and S-nitrosylated. It is noteworthy that so far, only these few residues have indications of regulation by Ox-PTMs and those sites can experience multiple modifications. The formation of disulfide bonds and RS-SG at these regulatory sites is negatively correlated with ATP hydrolytic activity, suggesting that these modifications cause profound conformational changes leading to the inactivation of ATP synthase complex. It is interesting to note that during CRT, the only ongoing clinically effective therapy for dysynchronous HF,³⁶ the disulfide bond at Cys294 has been found to be
reversed and replaced by SNO, which resulted in the recovery of ATPase activity. CRT can increase mitochondrial ATP synthase activity through the reversal of Cys cross-linking of its specific subunits, suggesting that CRT treatment enhances mitochondrial antioxidant defense systems or increases the cellular and mitochondrial reducing status. Reversible Cys Ox-PTMs of ATP synthase subunits are proposed to serve as a protective mechanism to prevent permanent oxidative damage to ATP synthase complex at the expense of decreased ATP production. However, in HF, this could preserve the cellular concentration of ATP, reducing the mitochondrial membrane potential (Δψ), thereby lowering the driving force for Ca²⁺ uptake into the mitochondrial matrix as suggested by Sun et al.³³ This would ultimately produce a protective phenotype in the heart.

Figure 3. Redox-sensor proteins and redox-switches. A, ATP synthase α-subunit cysteine (Cys) 294 functions as a redox-switch. In dyssynchronous heart failure (DHF), Cys294 of the α-subunit are S-glutathionylated, or interdisulfide bonds occur between Cys294 of α-subunits, as well as between Cys294 and Cys103 of the γ-subunit. Cys cross-linking inhibits its ATP production, leading to mitochondrial dysfunction. Cardiac resynchronization therapy (CRT) increases S-nitrosylation (SNO) of ATP synthase at Cys294 of the α-subunit by reverse Cys cross-linking, along with recovered ATPase activity. B, Regulation of actin polymerization through S-glutathionylation of Cys374 of actin. ROS indicates reactive oxygen species.

As Cys294 in the ATP synthase α-subunit is actively involved in various Ox-PTMs, including intermolecular disulfide bond formation, RS-SG, and SNO, each potentially competing with the others for occupancy of this residue, we have suggested that this amino acid functions as a redox-sensor.²⁶ Site-directed mutagenesis analysis confirmed that Cys294 is critical for the functionality of ATP synthase in vitro, and that it could play a critical role in redox-regulation of ATP production.²⁶ The precise mechanism by which these Ox-PTMs form has yet to be determined. Given that Cys294 is located on the surface ATP synthase where it is exposed to the environment and is surrounded by several basic amino acid residues,²⁶,⁶₅ it is likely to be deprotonated at physiological pH, making it a good candidate for oxidant attack. With increased oxidative stress in early stages of HF, it is conceivable that Cys294 could be oxidized to a sulfenic acid (although there is no evidence yet). This initial thiol modification of Cys294 or other PTMs could cause conformational changes that expose other Cys, such as Cys103 of ATP synthase γ-subunit to subsequent Ox-PTMs. This could result in the formation of an intermolecular disulfide bond or modification by glutathione to form a mixed disulfide bond. Nevertheless, all these modifications cause the reversible inhibition of ATP synthase activity and eventually lead to mitochondrial dysfunction in vivo. CRT treatment, which enhances the cellular antioxidant capacity by increased expression of proteins like peroxiredoxin 3,⁶₆ could reverse these dysfunctional disulfide bonds through the increased expression of the peroxiredoxin/thioredoxin pathway. Thus, this single modifiable Cys in ATP synthase seems to
act as a redox-sensor of local cellular redox-environment and modulate mitochondria ATP production.

**Actin**

Actin is a highly conserved protein in eukaryotic cells, and its 3 isoforms, α, β, and γ, share >90% amino acid sequence homology. All 3 actin isoforms can participate in the cytoskeleton when monomeric globular G-actin is activated to polymerize into filamentous F-actin on extracellular stimuli.68 In muscle, filamentous α-actin is also found in the contractile myofilament proteins where the F-actin thin filament (also comprising tropomyosin and the troponin subunits) interacts with the thick filament which is composed primarily of myosin, the enzyme that hydrolyzes the ATP required to drive muscle contraction.69

Actin polymerization is fundamental to many cellular activities, including motility, cytokinesis, and vesicle traffic.68 There is mounting evidence that the actin system (primarily the β and γ isoforms) is one of the most redox-sensitive constituents of the cytoskeleton.70 Recent redox-proteomics studies detected actin as the most prominent protein oxidized in response to exposure of cells to oxidants.71-74 In vitro, treatment of α-actin with 5 to 20 mmol/L H2O2 inhibited actin polymerization.75 In vivo, H2O2 treatment of cells has been found to cause cytoskeletal rearrangements, including F-actin fragmentation and an ammashment that correlated with gross cell morphological changes such as membrane blebbing.70

α-actin oxidation was also implicated in human diseases, such as HF and ischemia/reperfusion injury.76 In human end-stage HF, α-actin in the myofilaments was found to be a major target of protein carbonylation and is significantly correlated with both loss of viability and contractile dysfunction.77 Protein carbonylation is an Ox-PTM that occurs to many different amino acid residues (Pro, Arg, Lys, and Thr) and is considered to occur during higher levels of oxidative stress.78 Exposure of isolated rat hearts to 30 minutes of crisis, like in sickled red blood cells, there is an accumulation of Cys285-Cys374 intramolecularly disulfide bonded β-actin. This reduces actin filament dynamics and may help protect the cell from oxidative stress arising from normal oxidative metabolism and contribute to the cell’s general adaptive response to oxidative stress.81 Crystallization of H2O2 treated β-actin has shown that a Cys374-Cys374 intermolecular disulfide bond can form under such conditions,80 preventing polymerization. Crucially, the reduction of these oxidative Cys modifications likely takes place via the thioredoxin (Trx) system because Trx1 physically interacts with actin and has an important role in regulation of actin polymerization.82 Given this evidence, it seems actin Cys374 is in a prime position and functions as a redox-switch to help coordinate the response to oxidative stress.

**Other Examples of Proteins Modified by Ox-PTMs**

The list of proteins involved in cardiovascular diseases that undergo Ox-PTMs in Cys has been increasing during the last several years. For example, Ago et al and Oka et al84,85 have shown that the redox-state of histone deacetylase 4 (HDAC4) plays a role in cardiac hypertrophy, suggesting that the molecular mechanism by which Trx1 triggers nuclear translocation of HDAC4 through reduction of Cys residues in HDAC4, thereby inhibiting hypertrophy. HDAC4 at Cys667 and Cys669 are oxidized to form disulfide bonds in response to hypertrophic stimulation and this oxidation on Cys is responsible for cytoplasmic translocation. Trx1, an antioxidant protein, forms complex with TBP-2 (Trx binding protein-2) and DnaJb5, a heat shock protein. DnaJb5 is oxidized resulting in a disulfide bond between Cys274 and Cys276 in response to hypertrophic stimulation. Reduction of DnaJb5 by Trx1 through interaction with TBP-2 allows it to associate with HDAC4, whereas the oxidized form of DnaJb5 does not bind to HDAC4. Trx1 can reduce the disulfide bond of Cys667 and Cys669 in HDAC4 in the complex with TBP-2 and DnaJb5. This induces nuclear translocation of HDAC4, thereby attenuating hypertrophy through the suppression of key transcription factors such as nuclear factor of activated T cells.

Lovelock et al86 also recently showed a new example of the redox-state of Ox-PTMs in cardiac dysfunction. They demonstrated a significant increase of RS-SG of myosin-binding protein C with diastolic dysfunction, further demonstrating the necessity for understanding Ox-PTMs in the cardiovascular system.

**Experimental Tools for the Detection of Cys Ox-PTMs**

In 2001, Jaffrey and Snyder8 developed a technique for the detection of SNO termed biotin switch assay. Since then, multiple variations of this technique have been presented for the detection of not only SNO but also RS-SG, disulfide bonds, SSH, and SOH (Table). The workflow of the biotin switch assay includes (1) blocking all free thiols (nontarget), (2) specifically reducing the target Cys Ox-PTM (SNO-modified thiols are reduced by ascorbic acid), and (3) labeling the newly reduced thiol with biotin-HPDP(N-[6-(Biotinamido)
hexyl]-3′-(2′-pyridyldithio)-propionamide) which forms a disulfide bond with the available thiol. Since the initial description of the technique, other labeling reagents have been developed including those which alkylate target thiols. Once labeled, the SNO-modified proteins can be analyzed by gel-based assay/Western blot or be digested and specifically enriched and analyzed by mass spectrometry. This technique has also been modified to detect Cys Ox-PTMs other than SNO. Different reducing reagents are used to specifically reduce the different modifications. Reducing agents such as ascorbate, glutaredoxin, and m-arsenite have been used with SNO (also for SSH), RS-SG, and SOH, respectively.\(^8\)\(^7\)\(^8\) Depending on the goals of the study, there are a variety of reagents available, including those for general cellular detection or site identification and relative quantification. The latter has been achieved using labeling reagents such as isotope-coded affinity tag (ICAT)\(^8\).

More recently, our group has used cysteine tandem mass tag (cysTMT)\(^10\) to detect and determine individual Cys’s reactivity for SNO, whereas Kohr et al\(^8\)\(^9\) measured SNO occupancy in the myocardium with the same cysTMT.

In the case of sulfenic acid, dimedone chemistry has been applied to detect sulfenylated proteins.\(^15\)\(^16\) Dimedone is a cell-permeable small molecule and can trap sulfenic acid specifically by nucleophilic attack. Recently, Paulsen et al synthesized dimedone derivative, Dyn-2, which traps sulfenic acids efficiently in cells and can be used to enrich modified proteins using a click-chemistry reaction to an azide bead.\(^90\)

Unlike SNO or SOH, stable PTMs like SOH or SoH can be detected directly by mass spectrometry.\(^19\)\(^20\) Recently, Lo Conte and Carroll\(^91\) proposed a new 1-step reaction of SOH in physiological pH with potential utility for the detection of sulfinylated proteins to handle concerns with similar mass shift of SOH and sulfhydrated species.

**Future and Cross-talk Between Different Signaling Systems Within the Cell**

Emerging evidence suggests that Ox-PTMs may work in concert with other PTMs (such as phosphorylation, acetylation, ubiquitination, among others) to determine the ultimate biological outcome and cell phenotype (Figure 4). In this context, it seems that cross-talk between PTMs represent a complex regulatory network with characteristics of a dynamic code.\(^92\)

These networks should be fundamental to normal development and disease pathogenesis.\(^93\) In this context, the oxidation of the redox-sensitive Cys must lead to the activation or inactivation of the target enzymes (eg, kinase; Figure 4A) which in turn change the cellular PTM status (eg, phosphorylation) of their downstream targets. For instance, on treatment with \(H_2O_2\), the \(\alpha\) isoform of the cGMP-dependent protein kinase forms an intermolecular disulfide bond linking 2 subunits.\(^93\)\(^95\) The \(H_2O_2\)-induced protein dimerization directly activates \(\alpha\) isoform of the cGMP-dependent protein kinase and leads to the opening of Ca\(^{2+}\)-activated K\(^+\) (BKCa) channel. This participates in the subsequent smooth muscle hyperpolarization and dilation in the human coronary microcirculation.\(^95\)

Alternatively, it may be possible that other PTMs indirectly regulate the state of Ox-PTM (Figure 4B). For example, Trx1 has been suggested to play a role in denitrosylation. Yuan et al\(^96\) have demonstrated that Trx1 can be glycated by lipopolysaccharide and that glycation (not Ox-PTM) inhibited...
Trx1’s antioxidant action. The direct effect on SNO by glycation still needs to be elucidated but this type of indirect regulation will be promising.

In addition to the indirect activation effects, it is feasible that Cys Ox-PTMs could enhance or inhibit the addition of another modification (eg, acetylation, methylation, and phosphorylation) within the same protein to change its activity (cross-talk; Figure 4C and 4D). This is best exemplified by the forkhead transcription factor FoxO4, which plays a major role in the control of cellular proliferation, oxidative stress, and apoptosis. FoxO4 has been found to interact with the acetyltransferase p300/CREB-binding protein via an intermolecular disulfide link mediated through Cys477. Formation of the disulfide bond triggers acetylation of FoxO4 and represses transcriptional activity. Characterizing these types of interactions will be necessary for understanding how the cell detects and responds to various redox-signals and stresses.

In conclusion, the application of proteomic methods has increased the number of Ox-PTMs and the modified Cys residue, at least for some specific modifications. This collective data support the concept that Ox-PTMs are specific and selective. Furthermore, studies focused on specific protein as well as the occupancy of different Cys Ox-PTMs have increased our understanding of redox-regulation. It is also evident that redox-regulation is an important component of cardiovascular disease and that it is critical to unravel its complex regulation. In the meantime, the high selectivity inherent of Cys Ox-PTMs will provide novel therapeutic targets for the design of innovative drugs as a means to prevent and manage cardiovascular disease risk.

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Disclosures
None.

References


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