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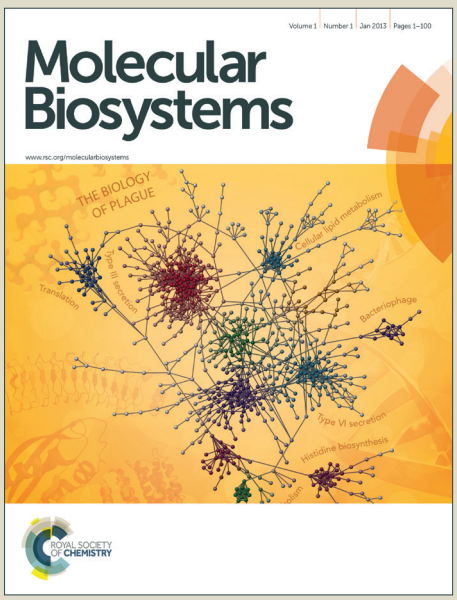

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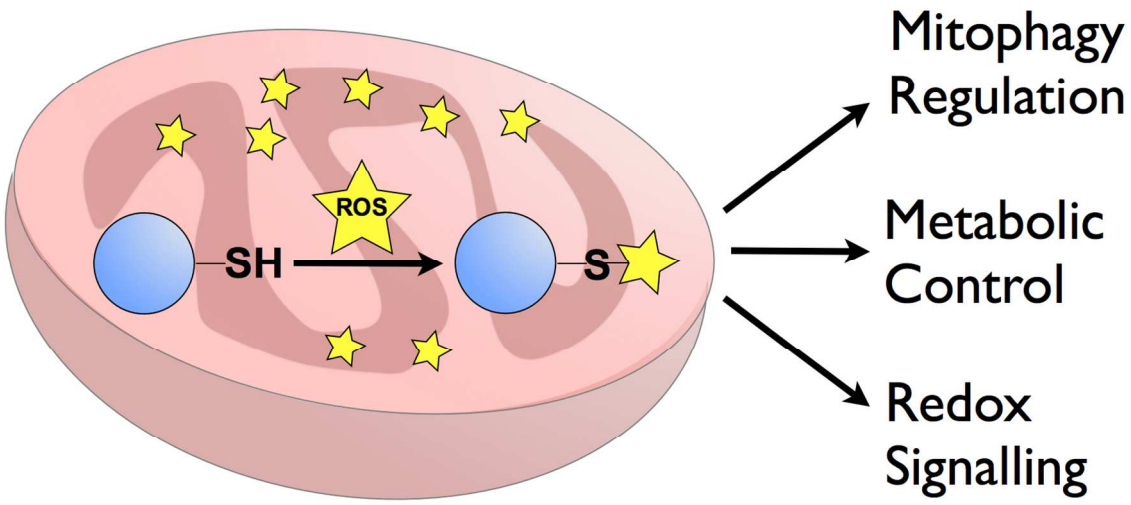

This review represents a novel look at the many sources, cysteine targets, and signaling processes of ROS in the mitochondria.

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Cysteine-Mediated Redox Signalling in the Mitochondria

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The mitochondria are critical mediators of cellular redox homeostasis due to their role in the generation and dissipation of reactive oxygen/nitrogen species (ROS/RNS). Modulations in ROS/RNS levels in the mitochondria are often reflected through oxidation/nitrosation of highly redox-sensitive cysteine residues within this organelle. Oxidation/nitrosation of functional cysteines on mitochondrial proteins serves to modulate protein activity, localization, and complexation in response to cellular stress, thereby controlling critical processes such as oxidative phosphorylation, apoptosis, and redox signalling. In this review, we describe mitochondrial sources of ROS/RNS, cysteine modifications that are triggered by increased mitochondrial ROS/RNS, and examples of key mitochondrial proteins that are regulated through cysteine-mediated redox signalling. We highlight recent advancements in proteomic methods to study cysteine posttranslational modifications. These tools will further aid in illuminating the important role of cysteine in maintaining and transducing redox signals in the mitochondria.

Introduction

The high reactivity of the cysteine sulfhydryl results in a wide range of post-translational modifications (PTMs) of this important amino acid, including oxidation,[1,2] nitrosation,[3,4] glutathionylation,[5] sulfhydration,[6] prenylation,[7] palmitoylation,[8] and adducts with lipid-derived electrophiles (LDEs);[9] as well as the less common, ubiquitination, phosphorylation, and methylation.[10] While some cysteine modifications occur enzymatically, a significant number are generated non-enzymatically by reactive oxygen and nitrogen species (ROS/RNS), such as superoxide ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), and nitric oxide ($NO^{\bullet}$). ROS/RNS were once thought to be toxic byproducts of oxidative metabolism that were harmful to the cell. While high levels of reactive compounds do indeed cause cellular damage through non-specific oxidation,[11] it is now known that production of ROS/RNS is regulated and controlled at various sites, including the endoplasmic reticulum (ER), the peroxisome, phagosomes, and the mitochondria. Cysteine modification through ROS/RNS were once thought to be artifacts of oxidative stress and highly damaging to proteins, but are now understood to be both reversible and functional. Examples of proteins that are regulated by cysteine oxidation or nitrosation include EGFR, PTP1B, PTEN, SHP2, USP1/2, Bcl-2, NF-kB, Complex I, and many others.[1,12-14]

Mitochondria act as the epicenter of cellular redox signaling by providing the principal sites of superoxide production for most cells, as well as known sites of nitric oxide accumulation. Critical cellular pathways such as redox homeostasis, oxidative metabolism and apoptosis are centered within the mitochondria and known to be regulated by ROS/RNS, primarily through modification of cysteine residues on mitochondrial proteins.[12-14] Known sites of cysteine oxidation/nitrosation in the mitochondria include the complexes of the electron transport chain (ETC), tricarboxylic acid cycle (TCA) enzymes, proteins in the mitochondrial permability transition pore (MPTP), mitochondrial fusion and fission proteins, and antioxidant enzymes.[12-14] However, due to limitations in current detection methods, it is likely that these proteins represent only a small fraction of total modified proteins. Challenges associated with identifying mitochondrial cysteine PTMs include: (1) the transient nature of these modifications that render them labile during cell lysis and mitochondrial isolation; and (2) the low abundance of mitochondrial proteins relative to cytosolic components, which often results in suppression of signals from mitochondrial species during analysis of whole cells or lysates. Further improvements in proteomic platforms are required to better understand the ubiquity of oxidative cysteine PTMs and the functional consequences of these modifications in mitochondrial function.

This review will focus on the generation, function, and identification of mitochondrial oxidative /nitrosative cysteine PTMs. The primary sites of mitochondrial ROS/RNS production will be

described, together with the cellular systems that scavenge these reactive species. Additionally, the common redox-regulated cysteine PTMs will be described, together with specific examples of the effects of these modifications on mitochondrial protein function.. Lastly, a brief description of the methodologies used to study oxidative cysteine PTMs and the existing limitations in applying these methods to study mitochondrial targets will be discussed.

Mitochondrial ROS/RNS Generation and Scavenging

Mitochondrial ROS sources

The specific sites and relative quantities of mitochondrial ROS production is controversial due to a variety of factors, including the transient nature of the reduced oxygen products, the variety of complex assays employed, the sources of purified enzyme, and if using whole cells or isolated mitochondria, the energetic and redox states of the mitochondria. A number of excellent reviews have discussed the myriad sites of mitochondrial ROS production.[12,15,16] Here we will briefly reiterate the most salient points.

Electron Transport Chain. The majority of the ROS produced by the mitochondria is generated by the ETC during oxidative phosphorylation (OXPHOS) (**Figure 1**). During oxidative metabolism, electron equivalents generated during glycolysis and the TCA cycle are stored as reduced NADH (E_m = -340 mV).[17] NADH is oxidized to NAD^+ by the first ETC complex (Complex I or NADH:ubiquinone oxidoreductase).[18] Electrons are transferred to a proximal flavin, shuttled along 8 Fe-S clusters, and into a terminal quinone, generating a reduced quinol. This quinol is freely diffusible within the inner mitochondrial membrane (IMM) and enters the quinone/quinol pool.[19] Complex III (ubiquinol-cytochrome *c* reductase), through a complex Q-cycle, transfers electrons from the reduced quinol pool to cytochrome *c* (cyt *c*), which then delivers electrons to Complex IV (cytochrome *c* oxidase), the site of O_2 reduction to H_2O (E_m = +810 mV).[20]

The energetically favorable process of electron transfer up the potential gradient is coupled to the active transport of protons across the IMM from the mitochondrial matrix to the mitochondrial inter-membrane space (MIM).[17,20] Complex I, III, and IV all pump protons against this gradient, resulting in a membrane potential ($\Delta\Psi$) across the IMM. This proton-motive force (PMF) is subsequently used by ATP synthase (Complex V) to drive the synthesis of ATP from ADP and P_i concurrent to proton transfer back across the IMM into the mitochondrial matrix. The function of ATP synthase is controlled by ANT (adenine nucleotide translocase) and UCP (uncoupling protein), which dissipate the membrane potential by returning protons to the matrix.[20]

A significant amount of ROS is thought to be produced by Complex I[16,21,22] and to a lesser extent by Complex III.[23] The most characterized site of ROS production is at the FMN cofactor at the proximal end of the Complex I electron-transfer wire.[24] An additional site, located at the terminal end of the electron-transfer wire of Complex I (either Fe-S cluster N2 or the quinone-binding site) is also thought to produce ROS.[21,25] Both of these sites would produce superoxide on the matrix side of the IMM. Complex III is also know to produce ROS, at both of its quinone binding sites,[26] which releases superoxide into the matrix and the MIM. Interestingly, while Complex IV reduces oxygen in four one-electron steps, the enzymatic mechanism appears to be finely tuned to prevent any significant superoxide generation at this site.[27]

Quinol Pool Reducers. In addition to Complex I, four other protein complexes lead to reduction of the quinone pool, including succinate dehydrogenase (Complex II, Sdh),[19,28] electron transfer flavoprotein:ubiquinol oxidoreductase (ETF:QO),[29] dihydroorotate dehydrogenase (DOD),[30] and mitochondrial glycerol-3-phosphate dehydrogenase (mGPDH).[31] All four of these enzymes produce various amounts of ROS, mainly at the site of their flavin cofactors. These enzymes release superoxide into the matrix, while mGPDH also releases superoxide into the MIM. All of these enzymes reduce the quinone pool under certain metabolic conditions (eg. high membrane potential), resulting in reverse electron flux through Complex I. This reverse electron flow and NAD^+ reduction produces very high levels of ROS, more so than NADH oxidation.[21,32]

TCA enzymes. A number of TCA enzymes, including pyruvate dehydrogenase (PDH), 2-oxoglutarate dehydrogenase (ODH),[33] and branched-chain keto-acid dehydrogenase (BCKDH) are also responsible for the production of superoxide.[34] While the rate of superoxide production from these TCA enzymes is often considered secondary to superoxide generated at the ETC, some studies suggest that under certain conditions (eg. high NADH and enzyme substrate concentrations), superoxide production from these dehydrogenases can constitute an even greater percentage of total mitochondrial superoxide production than Complex I.[34]

Additional Mitochondrial ROS Sources. The Mia40/Erv1 protein complex, like its ER-localized PDI/Ero1 counterpart, oxidizes disulfide bonds in nascent proteins that are shuttled through the protein translocase of the outer membrane (TOM) complex.[35] Reducing equivalents from Mia40/Erv1 can reduce cyt *c*,[36] but alternatively electron donation to oxygen results in hydrogen peroxide production.[37]

Another interesting source of mitochondrial ROS is the shuttling of electron equivalents from cytochrome *c* to p66shc, an isoform of SHC1, which localizes to the mitochondria upon phosphorylation.[38] $p66_{shc}$ interacts with and oxidizes cyt *c* leading to ROS production when levels of reduced cyt *c* are high due to low cyt *c* oxidase activity (eg. hypoxia).[39] This rapid and controlled ROS release leads to opening of the mitochondrial permeability transition pore (MPTP) and subsequent apoptosis.[40] This pathway represents one of the best described and truly regulated sites of ROS production within the mitochondria.

On the outer mitochondrial membrane (OMM), both monoamine oxidase A and B (MAO-A/B) produce ROS. MAO enzymes catalyze the oxidation of biogenic amines, concomitant with the release of hydrogen peroxide. The levels of ROS produced by MAO-A/B can be upwards of 50-times higher than complex III ROS production,[41] suggesting that under conditions of ischemia, aging, and exogenous amine oxidation, MAOs may be a significant source of ROS production at the mitochondrial surface.[42]

Dismutation of Superoxide. Superoxide is a highly reactive and unstable molecule that is rapidly dismutated to hydrogen peroxide. Dismutation occurs enzymatically through three superoxide dismutases (SODs); SOD1 (CuZn-SOD) in the cytosol, nucleus, and MIM, SOD2 (Mn-SOD) in the mitochondrial matrix, and SOD3 (Ni-SOD) in the extracellular milieu (**Figure 1**).[43] Superoxide does not cross the lipid barrier, so superoxide generated in the matrix is dismutated solely by SOD2 and superoxide generated in the MIM is dismutated by SOD1. SODs are highly expressed (~10 μM) and have rapid kinetics on the order of 2 x 10^9 $M^{-1}s^{-1}$.[44] The importance of SOD2 is highlighted by the neonatal lethality of the $SOD2^{-/-}$ mouse.[45] Additionally, superoxide can spontaneously dismutate to hydrogen peroxide (~10^5 $M^{-1}s^{-1}$),[46] which is the most likely outcome for superoxide in the absence of SOD and reactive species such as NO•.[47] These dismutation processes keep the concentration of superoxide in the low picomolar range.[48]

Compared to superoxide, whose short half-life and high reactivity make it a poor oxidative signaling molecule, hydrogen peroxide is well suited for this purpose. Hydrogen peroxide has a half life of ~1 ms, is membrane permeable, and has a steady-state

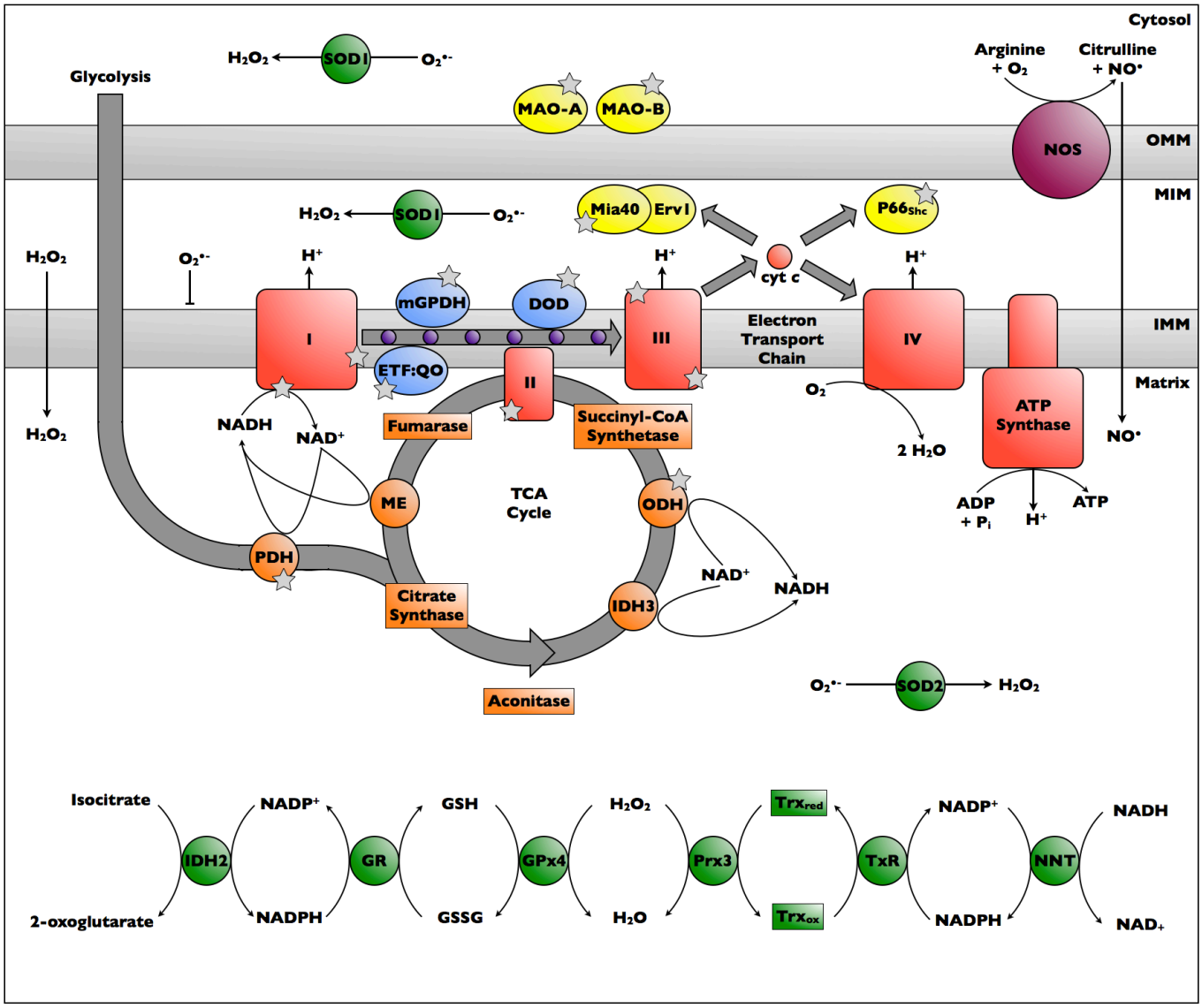

Figure 1: Sites of mitochondrial ROS generation. Reactive oxygen species (mainly superoxide) are generated at a number of sites within the mitochondria (grey stars), including the electron transport chain (ETC), tricarboxylic acid (TCA) cycle, and in the processes of oxidative disulfide bond formation in the mitochondrial intermembrane space (MIM). As electrons are pushed through the proteins of the electron transport chain (red round rectangles), protons are pumped across to the MIM, and ultimately oxygen is reduced to water by complex IV, all of which provides the driving force for ATP synthesis by complex V. During this process a number of sites in Complexes I, II, and III have the potential to transfer a single electron to free oxygen, generating a superoxide radical. Superoxide generation appears to occur mainly at the flavin and quinone binding sites in these structures. In addition to Complex I and II, a number of other proteins (blue ovals) deliver electrons to the quinol pool (small purple spheres) in the inner mitochondrial membrane (IMM). All of these proteins have also been identified as sites of ROS generation. A number of TCA cycle enzymes (orange circles and rectangles) are also sites of superoxide generation, including 2-oxoglutarate dehydrogenase and pyruvate dehydrogenase. Additional sites (yellow ovals) of mitochondrial ROS generation include, MAO-A and B, Mia40/Erv1 (the disulfide synthesis machinery of the mitochondria), and $p66_{shc}$. Superoxide is dismutated to hydrogen peroxide by superoxide dismutases (SODs) (green circles); in the mitochondrial matrix this occurs throught SOD2, while in the MIM and cytosol, SOD1 is the primary enzyme. Reduction of hydrogen peroxide to water is accomplished by both peroxiredoxin and glutathione peroxidase (green circles). Peroxiredoxins are rereduced by the NADPH-dependent thioredoxin/thioredoxin reductase system (green circles) while glutathione peroxidase reduces peroxide through concomitant oxidation of the glutathione pool, which can be rereduced by NADPH-dependent glutathione reductase (green circles). Both the Prx and GPx antioxidant systems rely on obtaining reducing equivalents from NADPH; NADH and NADPH are readily convertible through the actions of nicotinamide nucleotide transhydrogenase, connecting the metabolic processes of the TCA cycle to the antioxidant capabilities of the mitochondria. IDH2 is also able to generate significant levels of NADPH. Nitric oxide is known to be produced/transported into the mitochondria, most likely by the activities of a mitochondrial localized nitric oxide synthase (NOS) (purple large circle). Cyt *c*: cytochrome *c*, DOD; dihydroorotate dehydrogenase, ETF:QO; electron transfer flavoproteins:quinol oxidoreductase, GPx4; glutathione peroxidase 4, GR; glutathione reductase, IDH; isocitrate dehydrogenase, MAO-A/B; monoamine oxidase-A/B, ME; malic enzyme (malate dehydrogenase), mNOS; mitochondrial nitric oxide synthase, mGPDH; sn-glycerol-3-phosphate dehydrogenase, NNT; nicotinamide nucleotide transhydrogenase, ODH; 2-oxoglutarate dehydrogenase, PDH; pyruvate dehydrogenase, Prx3; peroxiredoxin 3, SOD; superoxide dismutase, Trx; thioredoxin, TxR; thioredoxin reductase.

concentration in the cell in the nanomolar to low micromolar range (at least 100 fold higher than superoxide). These properties enable hydrogen peroxide to diffuse farther and signal at a greater distance from its site of generation (~5 μm), which is significantly greater

than the diameter of a mitochondria.[48,49]

Mitochondrial RNS sources

In addition to ROS, cells contain RNS mostly in the form of nitric oxide ($NO^{\bullet}$). It is generally accepted that mitochondria contain bioactive $NO^{\bullet}$,[50,51] but the exact source of this $NO^{\bullet}$ is unclear. All three nitric oxide synthase enzymes (nNOS, iNOS, and eNOS) have been found in or associated with the mitochondria under certain conditions (eg. acute inflammation) (**Figure 1**).[52] None of the NOS enzymes contain identifiable mitochondrial targeting sequences and it is hypothesized that an N-terminal cleavage or splicing event triggers mitochondrial localization.[52] $NO^{\bullet}$ is generated in NOS enzymes by the reaction of molecular oxygen and arginine. Interestingly the K_m of oxygen for NOS is two orders of magnitude higher than that of Complex IV,[53] which is higher than usually present in the mitochondria.[54] Since $NO^{\bullet}$ is so unreactive, it is thought that the relevant cellular $NO^{\bullet}$ signaling molecule is likely N_2O_3,[55] generated by the reaction of $NO^{\bullet}$ with O_2. The high level of oxygen required to stimulate NOS-mediated production of $NO^{\bullet}$, likely favors N_2O_3 production. It is also possible that nitric oxide is synthesized outside the mitochondria and diffuses in. $NO^{\bullet}$ has a half-life of ~1-5 sec, depending on cellular conditions, and can diffuse a large distance across cellular membranes.[56] Alternatively, $NO^{\bullet}$ could enter the mitochondria as low molecular weight SNO compounds, such as cysteinyl-NO (CSNO) or nitrosoglutathione (GSNO).[56]

In addition to superoxide, peroxide, and nitric oxide, a number of other ROS/RNS species exist. Most of these additional species are extremely reactive and lead to significant damage to cellular components including proteins, DNA, and lipids.[48,57] It is unlikely that these molecules are used for signaling as they are far too damaging. The most common is the hydroxy radical, which results from Fenton chemistry that occurs when hydrogen peroxide and Fe-S clusters interact. In this process the cluster is often degraded and hydrogen peroxide is reduced by one electron to produce a hydroxy radical.[58] Peroxynitrite results from the radical-radical chemistry that occurs when superoxide and $NO^{\bullet}$ interact.[59] This intermediate is also extremely reactive and toxic to the cells.[60] A more interesting intermediate is hypochlorous acid (HOCl), which is produced enzymatically by myelo- and eosinophil-peroxidases that convert peroxide into HOCl and hydroxide.[61] HOCl has reactivity similar to hydrogen peroxide, but its kinetics with respect to thiol reactions are many orders of magnitude faster.[62] The relevance of hypochlorous acid as a signalling molecule is unclear, though its highly specific reactivity makes it a potential candidate for redox signalling.

Hydrogen Peroxide and Nitric Oxide Scavenging Systems

In addition to the localized production of ROS/RNS in mitochondria, this organelle also contains dedicated defense systems against detrimental increases in ROS/RNS levels (**Figure 1**). Hydrogen peroxide can be scavenged by two enzymatic systems, peroxiredoxin and glutathione peroxidase, of which the most efficient is the peroxiredoxin system. Peroxiredoxins (Prxs) exists as dimers, with two critical cysteine residues per monomer; the peroxidatic cysteine and the resolving cysteine.[63,64] Upon interaction with hydrogen peroxide, the peroxidatic cysteine is oxidized to a sulfenic acid, which generates an intermolecular disulfide with the resolving cysteine of the other monomer to release hydroxide. The reduced state of the dimer is restored by the thioredoxin/thioredoxin reductase (Trx/TrxR) system,[65] which ultimately derives reducing power from NADPH. Mitochondria contain two isoforms of peroxiredoxin; Prx3 and Prx5.[63] Prx3 is two orders of magnitude more efficient at degrading hydrogen peroxide,[63] while Prx5 reacts more efficiently with organic hydroperoxides, which are products of oxidative damage.[66]

The second enzymatic pathway for peroxide scavenging in the mitochondria is through glutathione peroxidase (GPx), which catalyzes the reduction of hydrogen peroxide concomitant with the oxidation of two molecules of glutathione (GSH) to GSSG. GSSG is then reduced to GSH by the NADPH-dependent glutathione reductase (GR).[67] Like peroxiredoxin, two GPxs are found in the mitochondria; Gpx1 is soluble and located in the matrix, while Gpx4 reduces lipid hydroperoxides.[68] Both enzymes are highly efficient at catalyzing the reduction of their respective substrates.[63]

While the function of both Prx and GPx appears redundant, Gpx1 knockdown results in increased sensitivity to oxidative stress.[69] The lethality of Prx3 deficiency has not yet been determined.[70] The Prxs are expressed at about 1-2 fold higher concentrations than Gpx1 and Gpx4, and are responsible for ~90% of all mitochondrial peroxide scavenging.[71] An additional enzymatic peroxide scavenging system is catalase. This enzyme is localized in peroxisomes and likely plays a negligible role in controlling ROS levels inside the mitochondria, though one study did detect catalase activity in rat heart mitochondria.[72]

Non-enzymatic systems, such as lipoate and α-ketoacids, also sequester peroxide and regulate mitochondrial ROS levels.[73,74] Lipoate, like other sulfhydryl groups, reacts rapidly with peroxide, which inactivates lipoate-dependent enzymes such as PDH and ODH.[75] α-Ketoacids, such as pyruvate, oxaloacetate, and 2-oxoglutarate also react with peroxide.[74] While the kinetics of these reactions are much slower than their enzymatic counterparts, the concentrations of these metabolic intermediates can be many orders of magnitude greater than either Prx or Gpx, suggesting a potential role for these cofactors and metabolites in mitochondrial ROS regulation.[76]

Currently there is limited knowledge of direct scavenging systems for $NO^{\bullet}$ in higher eukaryotes. In some bacteria and lower eukaryotes, $NO^{\bullet}$ is reduced by nitric oxide reductases (NORs), which are often coupled to the bacterial ETC.[77] In higher eukaryotes, $NO^{\bullet}$ can be cleared through a more complicated mechanism involving glutathione, which will be discussed later. $NO^{\bullet}$ can also be converted to NO_2 by the plasma multicopper oxidase, ceruloplasmin.[78]

Oxidative/Nitrosative Cysteine Modifications

Reactive Cysteines

Even though cysteine is one of the least abundant amino acids in eukaryotic cells (accounting for only 2% of the total amino acid content), it is one of the most highly conserved residues within protein sequences.[79] The cysteine sulfur is highly reactive due to the fact that it is large, polarizable, very electron rich, and capable of adopting multiple oxidation states.[80] This reactivity and electron-rich nature enables cysteine to function as an active-site nucleophile, a metal-binding ligand, or to react with other sulfhydryl groups to form disulfide bonds.[81] The reactivity of a cysteine is highly dependent on its ionization properties (i.e. its pK_a).[1] The pK_a of free cysteine is ~8.3, but the local environment around any given protein thiol can dramatically decrease this value.[82,83] Local features, such as positively charged amino acid residues, or the partial charges of backbone nitrogens and α–helix dipoles, will decrease cysteine pK_a.[84,85] An example is the catalytic cysteine of glutaredoxins (Grxs), which is located proximal to a lysine residue and the N-terminal end of an α–helix. This local microenvironment results in a depressed cysteine pK_a of 3.5 and 4.6 for Grx1 and Grx2, respectively.[86,87] In addition to the reactivity of the cysteine itself, the propensity for cysteine modification is also dependent on the

steric tolerance of the local protein environment.[88,89]

In the mitochondria, cysteine residues may be especially likely to harbor oxidative and nitrosative modifications, due to the redox and pH state of this organelle. Since protons are actively pumped out of the mitochondrial matrix and into the MIM, the matrix is alkalinized compared to "standard" cellular conditions. Likewise the MIM is more acidified. This results in a greater propensity for cysteine ionization. Additionally, depending on the respiratory state of the mitochondria, the proton gradient can collapse through diffusion of protons into the mitochondrial matrix, facilitated by UCPs or ANT. Depending on the pK_a of an individual cysteine, this could lead to a change in ionization state and a loss of reactivity, controlling the likelihood of fluctuations in cysteine modifications under different mitochondrial conditions.

In addition to local pH changes, the mitochondrial matrix is a highly reducing environment compared to the cytosol. The potential of the 2GSH/GSSG couple in the cytosol is -240 mV (even higher in some other organelles),[90] whereas in the mitochondria, these potentials have been calculated to fall between -280 and -340 mV (depending on experimental conditions).[91] This reducing environment favors the reduced state of cysteine thiols under resting conditions in the mitochondria. It has also been estimated that the concentration of protein thiols in the mitochondria is 60-90 mM,[92] higher by an order of magnitude than the concentration of GSH (5 mM). Therefore protein cysteine residues are the most concentrated thiol within the mitochondria, which renders them highly susceptible to oxidation/nitrosation when ROS/RNS levels are perturbed.

Oxidative Cysteine Modification

As described previously, due to the rapid kinetics of SOD, the most relevant oxidative species in the mitochondria is hydrogen peroxide, especially considering its reactivity with reduced cysteine thiols, prolonged half-life and membrane-diffusion capability. Peroxide signaling proceeds through generation of a cysteine sulfenic acid (RSOH), which occurs upon nucleophilic attack of peroxide by the cysteine thiolate (RS^-) to form RSOH and OH^- (**Figure 2A**).[1] Importantly, this is an oxidative reaction with respect to the sulfur atom, resulting in a change in oxidation state from -2 to 0. The reaction of peroxide with protein cysteine sulfhydryl groups is rather slow (~20 $M^{-1}s^{-1}$),[82] but can be increased by orders of magnitude (~10^8 $M^{-1}s^{-1}$) by the protein environment.[88,89] This increased reactivity is a result of local pH, thiol pK_a, and proximal steric factors. Likewise, the stability of the resulting RSOH group is also dependent on the local environment, and RSOH groups have low pK_a values, and likely exist in a highly reactive RSO^- state.[93,94] A number of studies have identified very stable sulfenic-acid groups, which are likely to be directly involved in redox signaling.[85,95] For example, the sulfenic acid of human serum albumin has a half-life of ~4 min.[96]

The fate of a protein sulfenic-acid is highly dependent on the local environment. The presence of a proximal cysteine or GSH facilitates rapid disulfide formation Alternatively, if no thiol group is available, reaction with a backbone amide nitrogen affords a cyclic sulfonamide.[97] Both disulfide and sulfonamide formation are reversed through the thioredoxin and glutaredoxin systems and protect the thiol from further irreversible oxidation.[98] Furthermore, the resulting conformational changes induced by these modifications may be relevant for regulation of protein function.

Lastly, excessively high peroxide concentrations result in further oxidation of the thiol. These oxidations occur with a significantly lower rate constant (~0.1-100 $M^{-1}s^{-1}$) compared to the initial oxidation.[88,99,100] The reaction of sulfenic acid with peroxide results in the generation of sulfinic acid (RSO_2H) and water. The pK_a of sulfinic acid is ~2, and will therefore exist as RSO_2^- in biological systems.[93,94,100,101] Sulfinic acid is not capable of reaction with reduced thiols, and is generally not susceptible to cellular reductants. It was originally thought that sulfinic acids were irreversible, but it is now known that sulfinic acids can be reversed enzymatically by the sulfiredoxin (Srx) system (discussed later).[102] The only other non-enzymatic fate for sulfinic acid is further oxidation by peroxide to sulfonic acid (RSO_3H). Sulfonic acids are still considered irreversible protein modifications and a sign of oxidative damage.

Nitrosative Cysteine Modification

As described previously, $NO^\bullet$ is rather unreactive, but modulates enzyme function by interacting with heme and metal prosthetic groups,[103] and forming protein S-nitrosothiols (RSNO). Yet, due to its reduced reactivity relative to peroxide, $NO^\bullet$ cannot react directly with reduced thiols.[104] Three possible mechanisms exist for RSNO formation: (1) reaction of a reduced thiol with an oxidized nitrogen species such as N_2O_3;[55] (2) one-electron oxidation of a reduced thiol to a thiyl radical, and subsequent radical-radical combination with $NO^\bullet$;[105] or, (3) reductive heme-nitrosylation and autotransfer to a nearby thiol (**Figure 2B**).[106] The formal oxidation state of the sulfur in RSNO is 0, similar to that of sulfenic acid, but in contrast, RSNO is not ionizable.[107] RSNO is susceptible to hydrolysis, whereby the product could be either sulfenic acid or the free thiolate,[108] depending on the sulfur pK_a (**Figure 2B**). Unlike sulfenic acid, RSNO contains two electrophilic atoms, the sulfur and nitrogen. Based on free thiol and HNO pK_a values, attack on the nitrogen is generally thought to be more favorable,[109] but if the electrophilicity of the sulfur group is increased, then attack on the sulfur may be favored.[110]

In the presence of another thiol, RSNO can undergo disulfide-bond formation or transnitrosation (**Figure 2B**). As with the hydrolysis reaction described above, it is believed that transnitrosation (attack on the nitrogen) is the more favored pathway. Increasingly, transnitrosation is viewed as a highly relevant mechanism for S-nitrosothiol formation,[4,111] and many S-nitrosation studies employ small-molecule $NO^\bullet$ donors such as GSNO, S-nitrosocysteine, and S-nitroso-N-acetyl-D,L-penicillamine (SNAP).[51,112-114] Highly abundant small molecule and protein thiols, such as GSH, thioredoxin (Trx), albumin, and hemoglobin have been proposed to be physiological transnitrosation reagents.[115-117] For example, SNO-thioredoxin 1 transnitrosates procaspase 3, which in turn transnitrosates X-linked inhibitor of apoptosis (XIAP).[116,118] The full extent of endogenous transnitrosation within the mitochondria is poorly understood, as the majority of studies involve *in vitro* nitrosation or addition of large excesses of small molecule SNOs.

The Role of Glutathione

Glutathione reverses cysteine oxidation and nitrosation and protects cysteines from further oxidative damage. As briefly described previously, both sulfenic acids and S-nitrosothiols react with GSH to form glutathionylated adducts of cysteine, and release of water or HNO respectively (**Figure 2B**). Alternatively, in the case of GSH reaction with RSNO, transnitrosation generates the reduced thiol and GSNO. Due to the high concentration of GSH in the mitochondria, reactions with glutathione are likely to play an important role in preventing irreversible oxidation of sulfenic acids to sulfonic acids.

Transnitrosation of GSH by a protein S-nitrosothiol appears to be the main pathway for RSNO reduction and reversal, due to the presence of GSNO reductase (GSNOR), which reduces GSNO to HNO and GSH.[119,120] GSNOR is highly specific for GSNO and GSNOR deficiency results in a significant increase in protein-SNO levels.[119,121] The GSNOR system thereby protects the cell from

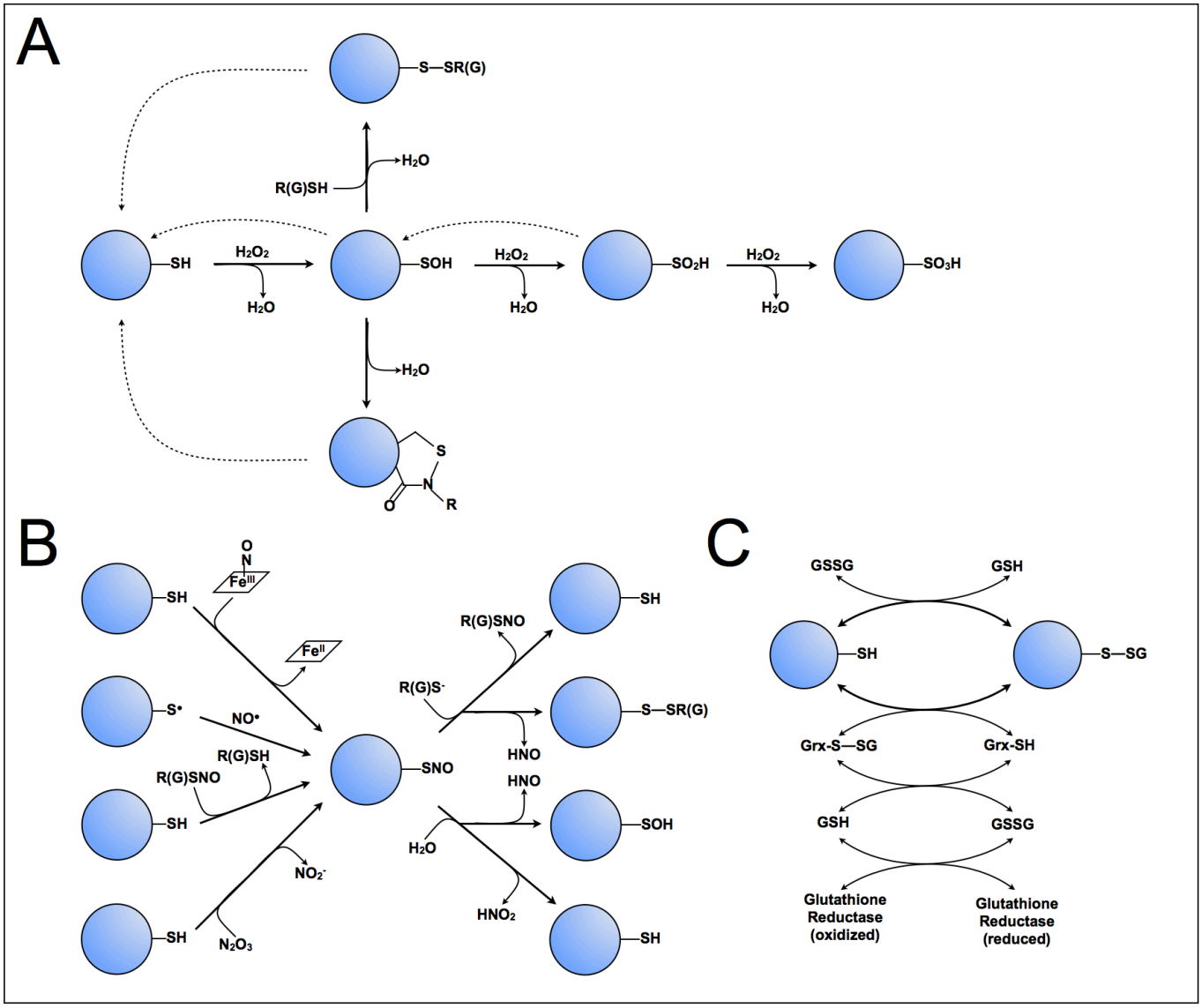

Figure 2: The range of oxidative/nitrosative cysteine post-translational modifications. A) Oxidative modifications of cysteine are the result of hydrogen peroxide reaction with a free thiolate; as additional equivalents of hydrogen peroxide react with the cysteine residue, more oxidized modifications occur, from sulfenic acid (SOH), to sulfinic acid (SO_2H), and finally sulfonic acid (SO_3H). Both sulfenic and sulfinic acids can be reduced through enzymatic reactions (dashed arrows). Sulfenic acids can also undergo further reactions with peptide backbone nitrogens to form sulfenamide adducts or with other protein or small molecule thiols to form disulfides, which are also enzymatically reversible. B) Nitrosative modifications occur through a number of distinct mechanisms that all involve nitric oxide. The fate of this S-nitrosothiol (SNO) modification depends on the relative reactivity of the nitrogen and sulphur electrophiles, but reaction with water can lead to either a sulfenic acid or a free thiolate, while reaction with a free thiol can either result in disulfide formation or transnitrosation. C) Glutathionylation of a cysteine occurs non-enzymatically from interaction with oxidized GSSG (dependent on the 2GSH/GSSG ratio), or enzymatically from the activities of glutaredoxins. S• indicates a thiyl radical species and S-SR(G) indicates a cysteine disulphide with another thiol or glutathione

excess nitrosation and subsequent protein damage.

Glutathione also reacts with free thiols by two general mechanisms, non-enzymatically and enzymatically (**Figure 2C**). Non-enzymatic glutathionylation proceeds via a thiol-disulfide exchange between a cysteine thiolate and GSSG, resulting in formation of RSSG and GSH.[122] This reaction is non-specific and occurs if GSSG is abundant. In the mitochondria, this reactionlikely occurs at a 2GSH/GSSG ratio of ~1.[123] This high ratio is indicative of oxidative stress, and non-specific protein S-glutathionylation serves a protective function, preventing irreversible protein oxidation. In general, the extent of non-enzymatic protein glutathionylation correlates with GSSG levels.

Enzymatic protein S-glutathionylation is highly specific, occurs under normal (non-oxidative stress) conditions, and is highly reversible. In the mitochondria, the glutathionylation and deglutathionylation of proteins is carried out by glutaredoxin 2 (Grx2).[124] The deglutathionylation reaction is much better understood relative to the glutathionylation activity of Grx2. Deglutathionylation proceeds by thiol-disulfide exchange between RSSG and an N-terminal Grx cysteine residue. The resulting Grx-SSG reacts with an equivalent of GSH to generate GSSG, which is reduced to 2GSH by glutathione reductase, an NADPH-dependent enzyme.[86] The reaction of Grx-SSG with GSH is rate limiting and build-up of this intermediate increases the glutathionylase activity of Grx2.[14] Interestingly, the Grx2 activity is regulated by an intermolecular [2Fe-2S] cluster.[125] Complexation of two monomers

of Grx2 with a [2Fe-2S] cluster renders the enzyme inactive. Disassembly of the [2Fe-2S] cluster during oxidative stress releases monomeric and active Grx2.[126] This mechanism for Grx2 activation under oxidative stress underscores the important protective function of glutathionylation.

Cysteine-mediated Redox Signalling

A large portion of cellular ROS/RNS is produced inside the mitochondria; it is therefore logical that cysteines onmitochondrial proteins will be targeted by these reactive species. The importance of reversible cysteine modifications to redox signaling within the cell has become much more appreciated over the years. A number of elegant studies have demonstrated the specificity of oxidative thiol modifications and the functional role of these modifications in the regulation of protein activity and signal transduction. These insights have been gained through advances in methods available to detect these modifications. However, the detection of endogenously oxidized cysteine residues, especially on mitochondrial proteins, is still complicated by a number of factors: (1) many of these modifications are transient with a short *in vivo* lifetime; (2) *in vitro* studies with exogenous and excessive oxidants can lead to non-specific and non-functional modifications; (3) mutagenesis studies are complicated by the difficulties in mimicking an oxidative modification with the available toolkit of natural amino acids; and, (4) *in vivo* studies are complicated by the fact that many proteins may be modified and contribute to an observed phenotype. Despite these limitations, there are several cases whereby cysteine modifications on mitochondrial proteins are well characterized to have functional consequences (**Table S1**). A subset of these functional mitochondrial thiol modifications is elaborated on below.

Mitochondrial Redox Sensing and Regulation

Peroxiredoxin. As described previously, peroxiredoxin acts to reduce cellular hydrogen peroxide. Upon oxidation of the peroxidatic cysteine to sulfenic acid in the dimeric protein, the resolving cysteine will form an intermolecular disulfide, which is reduced by NADPH-dependent thioredoxin (**Figure 3**). Peroxiredoxins are extremely efficient at catalyzing the reduction of peroxide, and the peroxidatic cysteine of Prx3 has an affinity for peroxide of $2x10^7$ $M^{-1}s^{-1}$ (5-7 orders of magnitude greater than most other reactive cysteines).[63] The cell is capable of maintaining the cytosolic concentration of peroxide below 100 nM,[127] which is too low to cause significant cysteine oxidation. Despite this, redox signaling is active in the mitochondria upon the induction of oxidative stress, suggesting the presence of a mechanism to transiently increase ROS levels. This "burst" of ROS is thought to result from peroxiredoxin hyperoxidation.

2-Cys peroxiredoxins from eukaryotes, such as the mitochondrial matrix peroxiredoxin (Prx-3), contain two unique

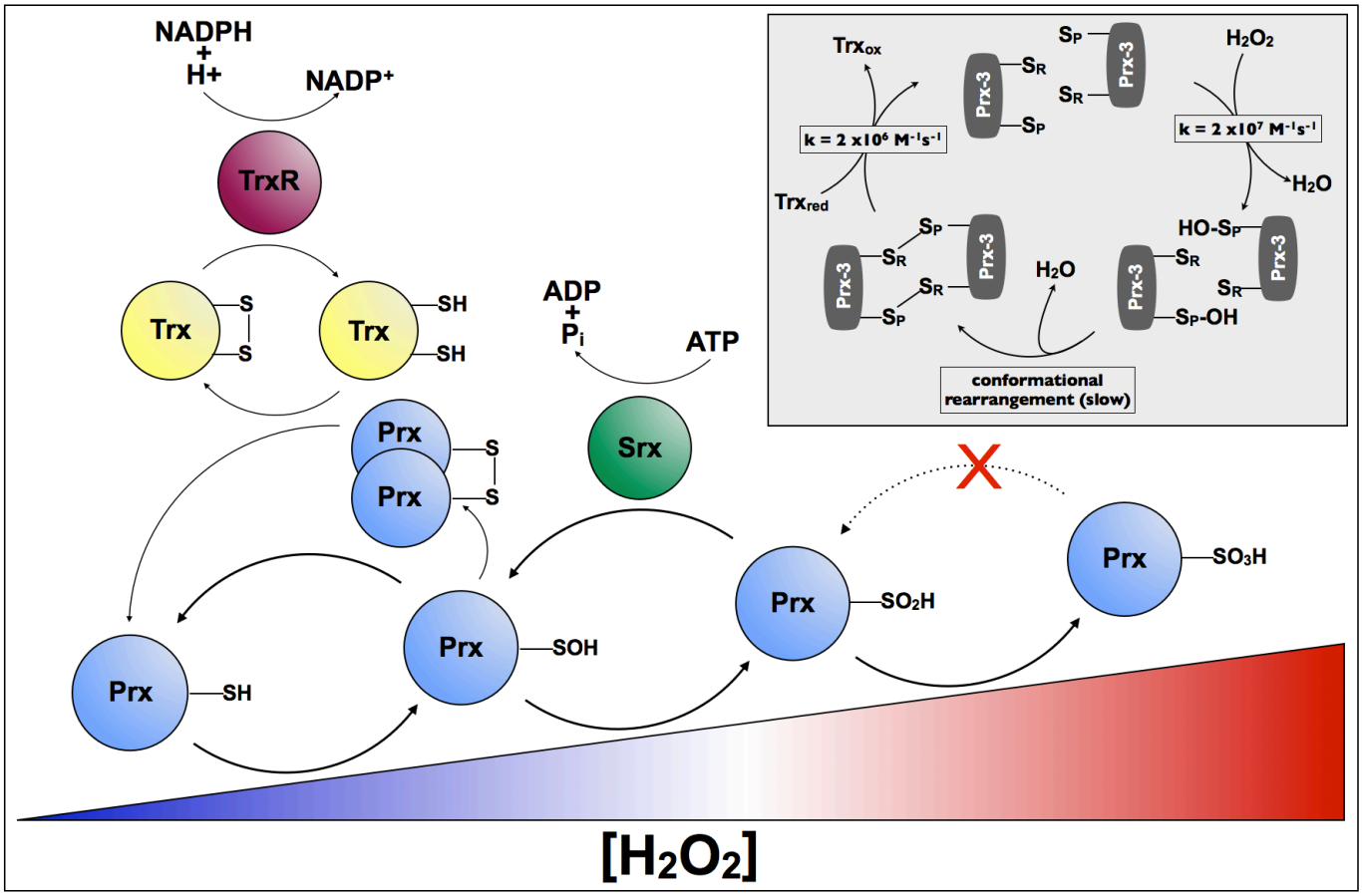

Figure 3: Hydrogen peroxide induced inactivation of peroxiredoxins to generate an oxidative burst. Under standard hydrogen peroxide levels, the peroxidatic cysteine of peroxiredoxin (Prx) reacts to form a sulfenic acid (SOH), which is converted to an intermolecular disulfide. Disulfide formation is relatively slow, due to the requirement for conformational rearrangement (inset). Disulfide reduction is promoted by the NADPH-dependent thioredoxin/thioredoxin reductase (Trx/TrxR) system. Upon increasing levels of H_2O_2, the peroxidase activity of peroxiredoxin is attenuated by hyperoxidation of the peroxidatic cysteine to form a sulfinic acid (SO_2H). Formation of this hyperoxidized species is possible due to the extended half-life of the sulfenic acid species. This inhibition of peroxidase activity allows for a buildup of ROS for signalling purposes. The sulfinic acid can be rereduced by ATP-dependent sulfiredoxin (Srx). At extreme levels of hydrogen peroxide, the peroxidatic cysteine is irreversibly over-oxidized to sulfonic acid (SO_3H).

sequence motifs (GGLG and YF) in the c-terminus of the enzyme near the resolving cysteine.[64,128] These sequences decrease the rate of disulfide formation and increase the lifetime of the sulfenic-acid intermediate. Under sufficiently high peroxide levels, the sulfenic-acid intermediate reacts with a second equivalent of peroxide to form sulfinic acid.[129] The rate constant for this reaction is $1.2x10^4$ $M^{-1}s^{-1}$, which in the case of Prx3 requires a local peroxide concentration of ~2 mM. Interestingly, mitochondrial Prx3 is 10 times less sensitive to hyperoxidation as compared to the cytosolic Prx2,[130] presumably because the mitochondria are routinely exposed to higher levels of steady-state peroxide. The sulfinic-acid form of the enzyme cannot be resolved by disulfide formation, and thereby renders the enzyme inactive. This inactivation at highlevels of mitochondrial peroxide is considered a "floodgate" mechanism, which allows for an oxidative burst to occur.[131] If peroxide levels surpass a certain threshold, they will overwhelm the usually very complete scavenging of peroxide by peroxiredoxin and allow for mitochondrial oxidative signaling. Mitochondrial sulfiredoxin, through an ATP-dependent mechanism can reverse this second oxidation reaction and recover the sulfenic-acid form of the enzyme (**Figure 3**).[132] Sulfiredoxin is a rather inefficient enzyme with rate constants around ~100 s^{-1},[133] which presumably further facilitates the build-up of hydrogen peroxide during this oxidative burst.

The oxidative burst resulting from peroxiredoxin inactivation allows the cell to activate antioxidant pathways to respond to the oxidative insult and restore redox homeostasis. In the case of insurmountably high levels of oxidative stress, the Prx/Srx system is overwhelmed and the peroxidatic cysteine is irreversibly oxidized to sulfonic acid. This final oxidation has a slow rate constant of 0.1-100 $M^{-1}s^{-1}$.[88,99,100] It is also worthwhile to note that the sulfiredoxin-mediated reduction of the sulfinic acid is impaired by low mitochondrial ATP, and the regeneration of reduced peroxiredoxin is inhibited by low NADPH levels.Therefore the activity state of these antioxidant systems is intimately tied to the overall metabolic state of the mitochondria.

MnSOD. MnSOD can be S-nitrosated or glutathionylated, which decreases enzyme activity[134] and invariably leads to an increase in mitochondrial ROS. The identity of the modified cysteine(s) and the cellular mechanism for this inactivation is not clear, but represents a potential avenue of mitochondrial cysteine-mediated redox control.

Control of Mitochondrial Metabolism

Since the ETC and the TCA cycle generate a large portion of mitochondrial and cellular ROS, it is not surprising that these enzymes are stringently redox regulated. Sites of cysteine oxidation, nitrosation, and glutathionylation have been identifiedin proteins within these two pathways. Addition of NO• donors, glutathionylating agents, and peroxide can result in changes in electron flow through the ETC and in metabolite flux through the TCA cycle. For example, addition of diamide to vascular smooth muscle cells increases intracellular GSSG levels, decreases respiration rates, and increases proton leakage. These effects are dependent on the concentration of diamide added,[135] with lower levels causing a transient effect and higher levels leading to irreversible outcomes. Redox-dependent cysteine modification has been studied most extensively in cardiac tissue following ischemia/reperfusion (I/R) injury, which deprives cardiac tissue of oxygen in the ischemic state and generates a ROS burst upon reperfusion (return of oxygenated blood to the tissue), leading to tissue damage.[136] Many proteins in the ETC or TCA cycle contain reactive cysteine residues that are modified during I/R injury.[137,138]

Complex I and the ETC. Exposure of heart mitochondria to oxidative stress results in decreased Complex I activity,[139] which becomes irreversible upon further oxidation. This irreversible fate is prevented by cysteine S-glutathionylation, which also decreases Complex I activity and ROS production,[139,140] but can be reversed by Grx-mediated deglutathionylation.[141] Cysteine residues in the 51 and 75 kDa subunits of the hydrophilic arm of Complex I are sites of S-oxidation or S-glutathionylation.[138] The 51 kDa subunit binds the FMN cofactor,[18] the site of the majority of Complex I superoxide production. The 75 kDa subunit is in close proximity to the 51 kDa subunit and contains two specific and relevant sites of cysteine modification, Cys_{531} and Cys_{704} (**Figure 4**).[139] Both sites are known to be S-glutathionylated at much lower levels of oxidants than other cysteine residues of the complex. S-glutathionylation of Cys_{531} and Cys_{704} may trigger a conformational change that blocks NADH binding to the hydrophilic arm of Complex I, limiting FMN reduction and electron flow through the respiratory chain.[139,141]

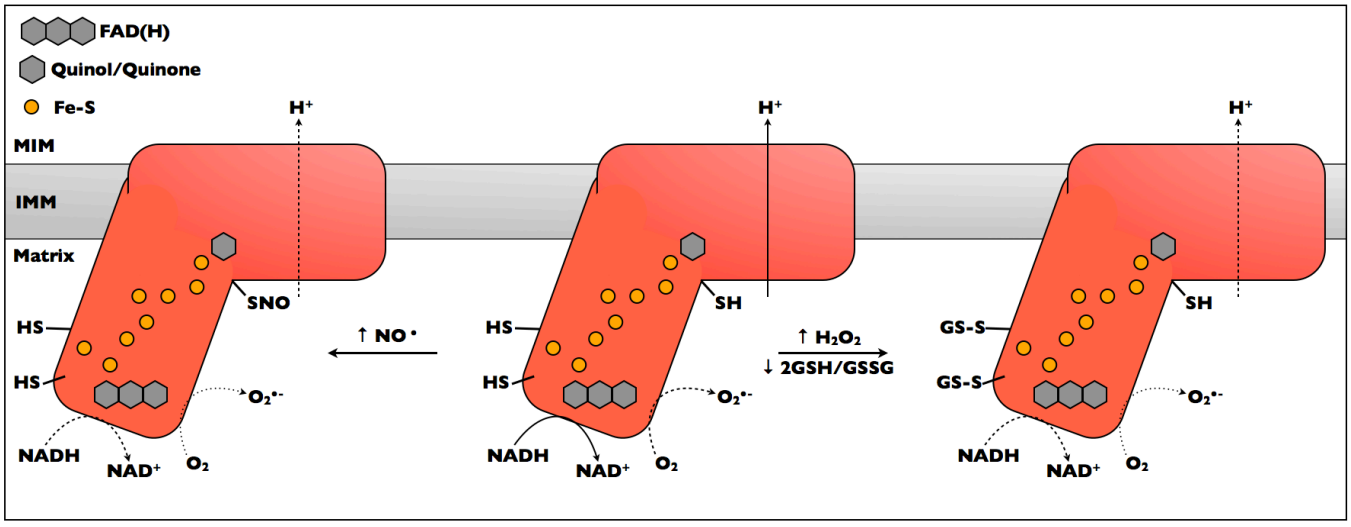

Figure 4: Protective effects of cysteine nitrosation and gluthionylation on Complex I. Complex I accepts electrons from NADH, transferring them along a wire from FAD to 8 iron-sulfur clusters and ultimately to quinone. Under standard conditions, low levels of ROS are generated at either the quinol or FAD sites, but under conditions where levels of NADH are high and electron flux is low, significant and damaging levels of ROS can be produced. Glutathionylation of two cysteine residues near the flavin binding site results in reduced enzymatic activity and a decrease in ROS generation, possibly by excluding NADH binding and electron transfer. Alternatively, nitrosation of a different cysteine prevents Complex I reactivation during ischemia/reperfusion, preventing a burst of ROS production from a rapid reactivation of the complex.

Alternatively, one study showed an increase in ROS generation of S-glutathionylated complex I,[142] but this finding is likely an artifact of the high GSSG concentration and unaccounted for complex I decomposition present in this study.[143]

Increased NO• levels render increased protection against I/R injury during myocardial infarction.[144,146,149] Although it is known that NO• can reversibly inhibit Complex I,[145-148] the direct mechanism of action was only recently elucidated. A study by Chouchani *et al.* demonstrated that S-nitrosation of Cys_{39} of the ND3 subunit of Complex I affords cardiac protection.[144] Using MitoB, and MitoSNO, a mitochondrial-localized peroxide sensor and nitrosating agent, respectively, it was shown that peroxide levels were highest after reperfusion and that S-nitrosation blocked this increase in peroxide. Proteomics experiments determined that only Complex I and Complex V were S-nitrosated, and interestingly, a single cysteine in Complex I was selectively nitrosated under ischemic conditions. It is known that Complex I undergoes a conformational transition from an active to a deactivated state under low oxygen or NADH conditions.[150] This deactivation is reversed upon increasing NADH levels. Cys_{39} was selectively S-nitrosated in the deactivated conformational state, and not in the active state. This cysteine is located near the ubiquinone-binding site, and is ideally situated to control Complex I activity. A current model is that S-nitrosation of Cys_{39} during ischemia prevents a rapid reactivation of Complex I, which thereby prevents to the generation of high levels of ROS and subsequent I/R injury (**Figure 4**).

Complex II (Sdh) is S-glutathionylated[138] at the 70 kDa subunit post I/R injury, whichin increases electron transfer and decreases superoxide production.[151] Deglutathionylation results in decreased electron-transfer activity. Superoxide production by Complex II results in a cycle of self-inactivation through tyrosine nitration.[152] Therefore, glutathionylation appears to be a protective mechanism to prevent damage from excess ROS production under ischemic conditions. Cysteine modification of Complexes III, IV, and V have also been identified,[138,145,153-156] but in general little is known about the physiological function of these, though oxidation/nitrosation of Complex III and V is also suggested to play a role in I/R injury.[138]

The TCA cycle. It is known that many TCA enzymes are targets for cysteine modification. Among these, aconitase,[157,158] ODH,[159] PDH,[160] and isocitrate dehydrogenase (IDH),[161] are all subject to S-glutathionylation. Aconitase is reversibly deactivated by glutathione during I/R injury, but this modification is not on a metal-binding cysteine, as no loss of [4Fe-4S] cluster binding is observed.[162] IDH2 can also be S-glutathionylated, leading to inactivation in HEK293 cells.[161] Interestingly, S-glutathionylation of IDH2 only occurs under high oxidative-stress conditions (2GSH/GSSG = ~1), which likely reflects on the critical role of IDH2 as a source of NADPH for mitochondrial anti-oxidant systems.[163] Both ODH and PDH can be oxidized on their lipoic-acid moiety, and S-glutathionylation of lipoic acid inactivates and protects these enzymes from irreversible oxidation.[164] Inhibition of ODH lowers NADH levels, which limits superoxide production by the ETC, and increases 2-oxoglutarate, which is itself an antioxidant.[165] As demonstrated, the redox regulation of metabolic enzymes is exquisitely controlled, and future work will fully illuminate how cysteine modifications on these enzymes work in concert to regulated mitochondrial metabolism.

Redox-Mediated Mitochondrial Quality Control

Other targets of oxidative and nitrosative cysteine modification includes proteins involved in mitochondrial dynamics and quality control. Examples include proteins involved in mitochondrial autophagy and redox control (Parkin and DJ-1), permeability (Cyclophilin D, Cofilin, and ANT), and fusion/fission events (Mfn1/2 and Drp1). While these proteins all act within unique physiological pathways, there is significant crosstalk between these mitochondrial functions. This section will focus predominantly on the regulation of Parkin (PARK2) and DJ-1 (PARK7), which are both implicated in the neurodegenerative disorder Parkinson's Disease (PD).[166,167] Other regulatory cysteine modifications associated with mitochondrial quality control will be touched on more briefly.

Parkin. Parkin is a known target of S-nitrosation that is associated with an autosomal recessive form of inherited PD. PD is characterized by a progressive loss of the dopaminergic neurons, resulting in a decline in motor functions, including slowness of movement, rigidity, and tremors.[168] Most cases of PD are idiopathic and thought to be a result of oxidative stress caused by Complex I dysregulation.[169] Additionally, there are cases of familial PD, which are caused by mutations in a handful of genes, including Parkin, DJ-1, α-synuclein, LRRK2, and PTEN-induced putative kinase 1 (PINK1).[166,167,170]

Parkin is an E2-dependent E3 ubiquitin ligase that transfers ubiquitin from an E2 ubiquitin-conjugating enzyme to a host of protein substrates (**Figure 5A**).[171] These ubiquitinated target proteins are subject to a variety of fates, including proteasomal degradation. Many Parkin substrates are cytosolic as Parkin is found predominantly in the cytosol where it regulates glucose metabolism, clearance of damaged proteins due to dopamine oxidation, and dopamine metabolism.[172] Additionally, a number of mitochondrial substrates of Parkin are known, including HSP70, TOM subunits, Mfn1/2, as well as mis-folded DJ-1.[173] A greater understanding of the targets of Parkin is needed to better understand the role of Parkin's protective functions.

It has been shown that Parkin can be localized to the mitochondria by mitochondrial depolarization, where it promotes elimination of damaged mitochondria through mitophagy.[174] Mitophagy is a mechanism by which the cell evades needless cell death, explaining the neuroprotective function of Parkin. The mechanism of Parkin recruitment to the mitochondria has been well studied and involves phosphorylation of Ser_{65} of Parkin by PINK1 (**Figure 5A**).[175] PINK1 is targeted to the mitochondria by a localization signal, and tethered to the OMM by an N-terminal helix. In healthy mitochondria, proteolysis of the N-terminal helix results in loss of mitochondrial localization. Under conditions of mitochondrial depolarization,[176] PINK1 accumulates at the mitochondrial surface and recruits Parkin, which results in ubiquitination of OMM proteins and subsequent mitophagy.

A recent study by Ozawa *et al.* demonstrated that while Ser_{65} phosphorylation controls mitochondrial localization of Parkin, it is actually S-nitrosation that regulates Parkin activity (**Figure 5A**).[177] Parkin S-nitrosation increased when oxidative phosphorylation was uncoupled or Complex I was inhibited, resulting in mitochondrial depolarization. Interestingly, S-nitrosation increased Parkin activity as monitored by autoubiquitination. The site of S-nitrosation was identified as Cys_{323}, which is neither the active site Cys_{431} nor the cysteine residues involved in zinc binding. Protein S-nitrosation was not observed in the C323S or C323A mutants and no increase in autoubiquitination activity could be detected in either mutant upon GSNO treatment. Lastly, overexpression of the C323S mutant in GSNO-treated HeLa cells led to decreased mitophagy relative to over-expressed wild-type Parkin. This result held true even when endogenous NO levels were modulated using the NOS inhibitors L-NNMA (N-monomethyl-L-arginine) and L-arginine. While additional work is needed to understand the underlying biochemical mechanism by which Parkin S-nitrosation results in increased activity, this study provides a link between increased S-nitrosation in PD patients and the role of mitochondrial function in

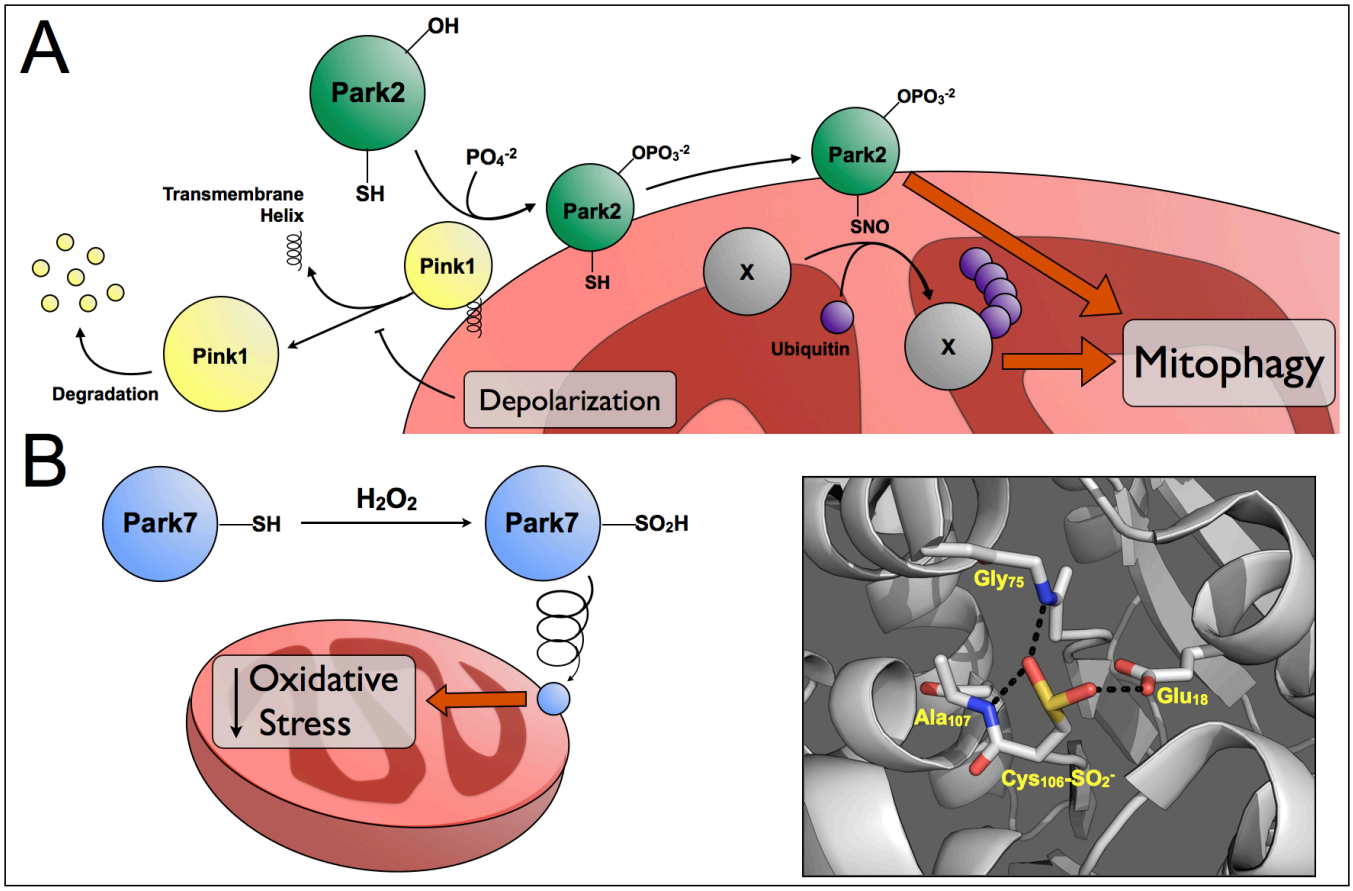

Figure 5: Nitrosation of Parkin and oxidation of DJ-1 modulates protein function. A) Parkin (PARK2) is localized to the mitochondria upon mitochondrial depolarization due to phosphorylation by PINK1, which is tethered to the mitochondria by a transmembrane helix. Under normal conditions this transmembrane helix is cleaved and PINK1 is not retatined at the mitochondria, but depolarization inhibits this cleavage event. Nitrosation activates Parkin activity, resulting in ubiquitination of a host of mitochondrial proteins leading to mitophagy. B) DJ-1 is a soluble cytosolic protein, which is recruited to the mitochondria by oxidative stress by oxidation of a single cysteine residue to a sulfinic acid that is stabilized by a nearby glutamic acid residue (inset). Oxidation results in mitochondrial localization, which eventually leads to a decrease in oxidative stress.

neurodegeneration.

DJ-1. DJ-1 (PARK7) is a cytosolic protein of obscure function that localizes to the mitochondria.[178,179] As with Parkin, DJ-1 is known to be neuroprotective, and increased DJ-1 levels protect cells against damage from oxidative stress.[178,180] DJ-1-deficient cells display altered mitochondrial morphology and increased ROS production.[181] Interestingly DJ-1 functions upstream of Parkin/PINK1, as DJ-1 negatively regulates Parkin localization to the mitochondria,[182] presumably by preventing PINK1-dependent phosphorylation. This presents an interesting dynamic between these three proteins that has still yet to be fully characterized. The function of DJ-1 is unknown, but it is suggested to be involved in copper transport,[183] chaperone activity,[184] ROS scavenging,[178,179] and protease or glyoxylase activity.[185,186] DJ-1 localizes to the mitochondria upon oxidation and this mitochondrial localization relieves oxidative stress by lowering ROS levels via an as yet uncharacterized mechanism (**Figure 5B**).[178,179]

Biophysical studies of DJ-1 have illuminated the mechanism of cysteine oxidation. Cys_{106} is oxidized preferentially over two other cysteine residues, Cys_{46} and Cys_{53}, which are less conserved.[187] In bacterial homologs, Cys_{106} is in a catalytic triad critical for protease activity, though this triad is absent in mammalian DJ-1.[185] Interestingly, sulfinic acid is the preferred oxidation state for this residue, which is a result of a strong hydrogen bond (~2.5 Å) between one of the sulfinic-acid oxygen atoms and a nearby glutamic-acid residue (Glu_{18}) (**Figure 5B - inset**).[178] Further oxidation to sulfonic acid is unfavorable due to a steric clash with a nearby histidine residue. In addition to the sulfinic acid found in Prx, DJ-1 provides one of the only other well-studied sulfinic-acid modifications. Elegant mutagenesis studies demonstrated that sulfinic-acid formation is required for the protective functions of DJ-1. An E18D mutant is not protective, presumably due to reduced stabilization of the sulfinic acid, while a C106D mutation, which mimics a sulfinic acid, retains its protective function.[188] The mechanism by which sulfinic-acid formation leads to mitochondrial localization is unclear and requires additional biochemical studies.

Mitochondrial Permeability. The mitochondrial permeability transition pore (MPTP) is involved in the release of apoptotic factors into the cytoplasm, collapsing proton and nutrient gradients, and mediating cell death during I/R injury.[189] The exact structure and subunit composition is still a matter of debate, but it is thought that diverse mitochondrial proteins such as $VDAC_1$, ANT, P_i Carrier and Cyclophilin D (CypD) may be involved. Interestingly, most of these proteins are known to contain various cysteine PTMs. Studies suggest the Cys_{160} sulfenic acid-mediated disulfide bond formation between Cys_{160} and Cys_{257} in ANT is required for MPTP pore formation.[190] Adenine nucleotide binding, can block the Cys_{160} residue preventing oxidation and MPTP formation. It has also been demonstrated that ANT is maintained in a S-glutathionylated state in rat cortex mitochondria.[191] CypD, the only component of MPTP

known to be indispensible for pore opening,[192] is known to be S-glutathionylated at Cys_{203},[193] preventing pore opening. Finally it has also been shown that cytoplasmic oxidation of the actin-binding protein, cofilin, leads to formation of two intramolecular disulfide bonds, which result in mitochondrial localization of cofilin and activation of the MPTP.[194]

Mitochondrial Dynamics. Both mitochondrial fission and fusion events are regulated by cysteine modifications. Mitochondrial fusion is mediated by the OMM GTPases, mitofusin 1 and 2 (Mfn1/2). Moderate (sub-lethal) levels of oxidative stress results in S-glutathionylation of Mfn2 and subsequent hyperfusion of mitochondria.[195,196] It is thought that mitochondrial fusion may alleviate local redox stress through a sharing of antioxidant defense systems. Alternatively, the dynamin family of GTPases, Drp-1, has been shown to be specifically S-nitrosated on one of its nine cysteine residues (Cys_{644}).[113,197] S-nitrosation of Drp-1 leads to increased mitochondrial fission and fragmentation in neurons.

Mitochondrial Proteomics of Cysteine Modification

The functional and regulatory mitochondrial cysteine modifications discussed previously likely represent a fraction of the physiologically relevant thiol modifications inside the mitochondria. Here, the current state of redox proteomics will be briefly examined together with the limitations in adapting these methods to study mitochondrial-modified thiols. The use of thiol-reactive probes to detect cysteine reactivity and/or cysteine modifications using qualitative gel-based or quantitative liquid chromatography/mass spectrometry (LC/MS)-based proteomics have been reviewed extensively.[12,198] For gel-based studies, labeling with a fluorescently tagged cysteine-reactive electrophile (iodoacetamide (IAM) or N-ethylmaleimide (NEM)) provides high sensitivity for thiol visualization using 1- or 2-D polyacrylamide gel electrophoresis (PAGE).[199] The difficulties inherent in gel-based proteomics are that subsequent MS identification is tedious and is not amenable to high-throughput analysis or the study of low-abundance proteins.[194,198,200] For these reasons the main focus of this discussion will be on LC/MS-based proteomics.

Cysteine modifications are detected by one of two general strategies: (1) directly, in which a probe specifically reacts with a single cysteine modification; or, (2) indirectly, in which free thiols are first blocked, the specific modification is reversed, and a second reagent is used to "capture" the newly freed thiol. While direct modifications are preferred, there are few established chemical reactions that provide the needed selectivity and rapid kinetics. Here, three commonly applied LC/MS methods will be described; (1) thiol-labeling strategies (e.g. oxICAT) that indirectly examine all oxidative cysteine modifications; (2) the Biotin Switch Technique (BST) and d-switch techniques, which are indirect methods to capture cysteine-SNO modifications; and (3) dimedone-based probes, which provide a direct labeling method for sulfenic acids.

Cysteine-Labeling Methodologies

The isotope-coded affinity tag (ICAT) methodology relies on the use of two or more isotopically distinct biotinylated-IAM probes, that differentially label reduced thiols from distinct proteome samples.[201] An extension of the ICAT methodology known as oxICAT is used to monitor the oxidation state of a thiol within a single proteome sample, rather than between two samples. One isotopic tag is used to label free thiols, following a reduction with DTT or TCEP, a second isotopic tag is used to label newly exposed reduced thiol groups (**FIgure 6A**).[202,203] This technique is an indirect method, and can only be applied in cell lysates due to the necessity to denature all proteins for complete capping of reduced cysteines. This limits the scope of this method since sulfenic acids and nitrosothiols are generally too unstable to survive cell lysis/homogenization.[204] In a related method, an iodoacetamide-alkyne (IA) probe is used to selectively tag a subset of reactive cysteines in the proteome. These IA-tagged proteins are then appended to isotopically labeled biotin tags for enrichment and MS-based identification and quantification.[205] In contrast to the oxICAT method, the IA probe can be applied in living cells and has the added advantage that proteins can be profiled in their native state, thereby maintaining the unique protein microenvironments that mediate cysteine pK_a, and enriching for the subset of reactive cysteines that are more likely to be susceptible to PTMs.

The BST for labeling SNO modifications is another indirect method,[206] in which reduced thiols are labeled with methylmethane thiosulfonate (MMTS) and then ascorbate is used to preferentially reduce SNO (**Figure 6B**). The resulting thiols can be captured by biotin-HPDP (N-[6(biotinamido)-hexyl]-3'-(2'-pyridyldithio)-propionamide) and enriched for MS studies.[114,115,207] An alternative technique, d-switch, utilizes two isotopically distinct NEM probes (d_0NEM and d_5NEM) to label the initially reduced thiols and the ascorbate-reduced SNO thiols.[208] Like oxICAT, both BST and d-switch must be performed in denatured lysates to successfully block all of the reduced thiols.

The most established direct-labeling probe is dimedone, which reacts very specifically with sulfenic acids.[198,209-211] Dimedone is not known to have reactivity with thiols, sulfinic acid, or other common biological functional groups, rendering it highly specific for sulfenic acids (**Figure 6C**). Commonly used dimedone probes include azido (DAz-1/2) and alkyne (DYn-1/2) versions[210,212] which are cell permeable, and can be coupled to biotin or fluorophores by Staudinger ligation[213] or Huisgen [3 + 2] cycloaddition.[214] Additional heavy versions of both the DAz and DYn probes have been generated for MS-based quantification.[215]

Proteomic Techniques for the Mitochondrial Proteome.

The application of these methods to identify cysteine modifications specific to the mitochondria is limited by a number of factors: (1) the instability of modifications once cells are lysed and treated; (2) the low abundance of mitochondrial proteins compared to the total protein content of the cell; and, (3) the current inability of cell-permeable probes to localize to the mitochondria. To gain a better understanding of the oxidative and nitrosative processes that occur within the mitochondria, these limitations must be overcome.

The effect of cell lysis on cysteine modifications is especially important for mitochondrial proteins considering that the redox state and pH level of the mitochondria can be markedly different than that of the cytosol. As described previously, the matrix of the mitochondria is a highly reducing environment, which also favors thiol ionization, which will be disrupted by cell lysis. In addition, changes in the local concentrations of H_2O_2, $NO^{\bullet}$, and glutathione due to cell lysis, will affect the extent of cysteine modification. Attempts to combat this loss in modifications utilize immediate TCA precipitation or incubation with ROS-metabolizing enzymes.[202,211]

The concentration of mitochondrial proteins can vary drastically by cell type. In some tissues, such as liver, mitochondria account for 1/5th of the cellular volume, while other tissues have a much lower concentrations of mitochondria per cell.[216] Regardless, mitochondrial proteins constitute a minor fraction of the total cellular protein. Mitochondrial proteins can be dramatically enriched by isolation of intact mitochondria from whole cells.[217] This process can be very time consuming, especially for the isolation of highly pure, actively respiring mitochondria, and ideal preparations can vary depending on cell type. Even though actively respiring, functional mitochondria can be isolated and treated with cell-

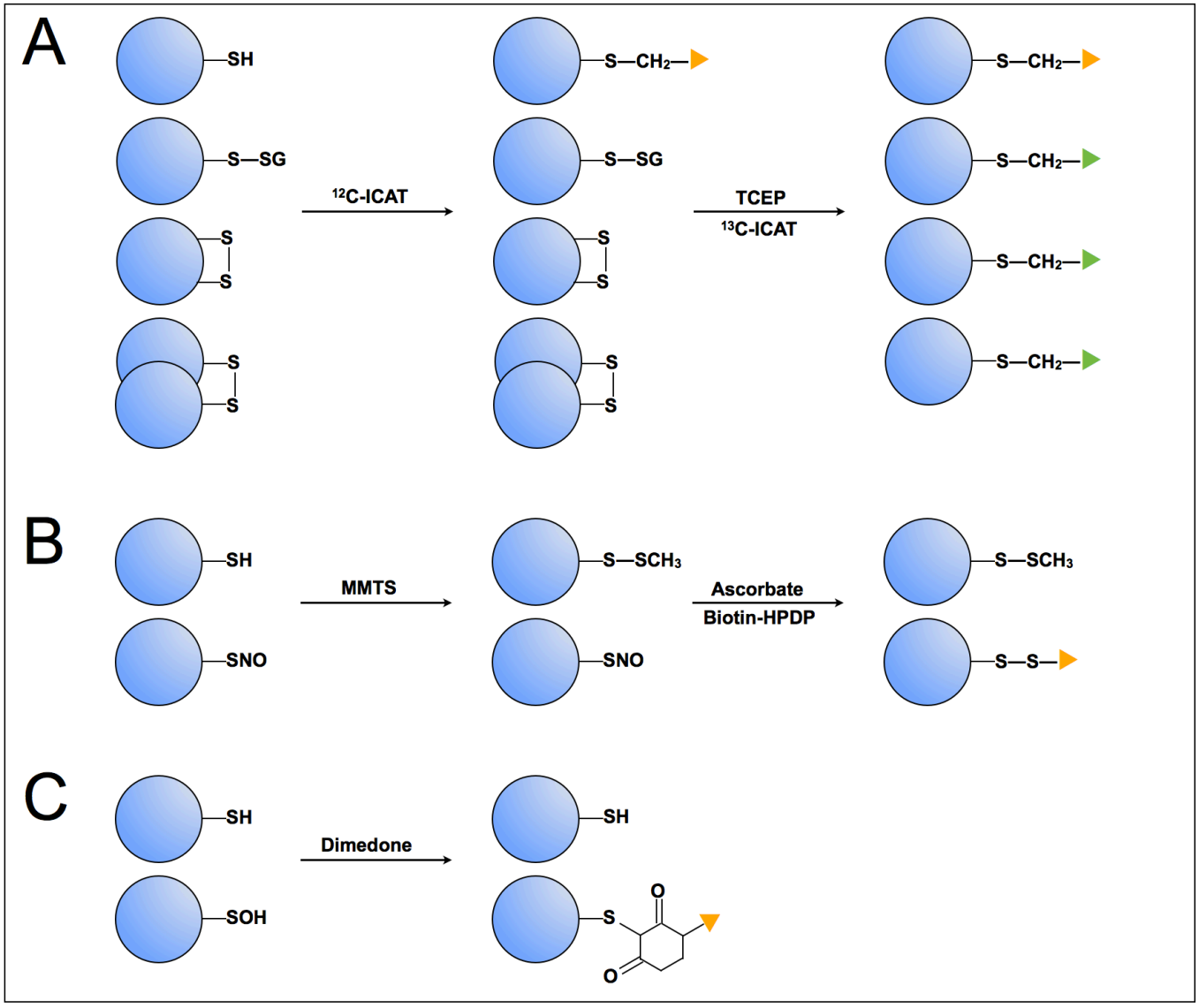

Figure 6: Proteomic techniques for the analysis of oxidative and nitrosative cysteine PTMs. A) The oxICAT (isotope-coded affinity tag) method differentially labels free thiols versus oxidized thiols within a proteome sample. Initial capping of free thiols with a 'light' isotopic tag (yellow triangle) is followed be reduction and capping of the previously oxidized thiols with a 'heavy' isotopic tag (green triangle). B) The Biotin switch technique (BST) involves capping all free reduced thiols with MMTS (methylmethane thiosulfonate), followed by ascorbate treatment to preferentially reduce nitrosothiols. These newly exposed thiol groups are then capped with biotin-HPDP ((N-[6(biotinamido)-hexyl]-3'-(2'-pyridyldithio)-propionamide) for enrichment. C) Sulfenic acid species can be directly labelled with dimedone. This probe is very specific for sulfenic acid and shows no reactivity towards free thiols or other oxidized cysteine species.

permeable probes, this is still a poor mimic of live-cell conditions.

One promising direction for the study of mitochondrial cysteine modifications is the use of targeted-probes, which will preferentially localize to the mitochondria. Current cell-permeable probes tend to localize in the cytosol, and therefore underrepresent the mitochondrial proteome. Several methodologies exist to localize small molecules to the mitochondria,[218] and should prove amenable to LC/MS-based proteomics studies. Unfortunately, an organelle-targeting approach would not be feasible with methods like oxICAT and BST, due to the reliance on complete capping of free cysteines prior to reversal of cysteine modifications. However, for probes such as IA, DAz and DYn, organelle targeting could be a promising approach to increase coverage of mitochondrial proteins.

Conclusions

As surveyed in this review, mitochondria are highly redox-active cellular organelles with numerous sites of ROS/RNS production and dedicated redox-defence systems to regulate the levels of these oxidants. As a result, many protein functions within the mitochondria are fine-tuned to respond to changes in local redox state. This regulation is often mediated through cysteine residues that are uniquely tuned to undergo oxidative and nitrosative modifications. These thiol-based redox modifications mediate mitochondrial redox state, metabolic function and protein homeostasis in response to changes to ROS/RNS levels. We have summarized several well-characterized cysteine modifications that regulate critical aspects of mitochondrial function. However, due to the prevalence of reactive cysteines on mitochondrial proteins, we hypothesize that there are myriads of cysteine PTMs within the

mitochondria that are yet to be revealed. Identification of these sites of cysteine oxidation is hindered by the lack of technological platforms to characterize these highly unstable and low-abundance modifications selectively on mitochondrial proteins. Recent advances in chemical-proteomic techniques to study cysteine modifications have facilitated a deeper study into cellular proteomes. Further adaptations to these existing technologies to selectively label, enrich and identify modified cysteines within the mitochondria, will likely illuminate a diverse array of redox-regulatory mechanisms within this organelle.

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Notes and references

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Electronic Supplementary Information (ESI) available: [A list of mitochondrial proteins with known cysteine oxidative, nitrosative, and/or glutathione modifications is included as Table S1]. See DOI: 10.1039/b000000x/

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