Review

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Thiol switches in mitochondria: operation and physiological relevance

Abstract: Mitochondria are a major source of reactive oxygen species (ROS) in the cell, particularly of superoxide and hydrogen peroxide. A number of dedicated enzymes regulate the conversion and consumption of superoxide and hydrogen peroxide in the intermembrane space and the matrix of mitochondria. Nevertheless, hydrogen peroxide can also interact with many other mitochondrial enzymes, particularly those with reactive cysteine residues, modulating their reactivity in accordance with changes in redox conditions. In this review we will describe the general redox systems in mitochondria of animals, fungi and plants and discuss potential target proteins that were proposed to contain regulatory thiol switches.

Keywords: glutathione; hydrogen peroxide; mitochondria; NADPH; reactive oxygen species; redox regulation; ROS; signaling; thiol switch.

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Introduction – mitochondria contain two distinct redox networks

Mitochondria are essential organelles of eukaryotic cells. They produce not only the bulk of cellular energy in the form of ATP, but they also generate numerous important metabolites and cofactors, and they serve as critical signaling stations that, for example, integrate cellular signals to initiate apoptosis. In turn, mitochondria also communicate their metabolic and fitness state to the remainder of the cell to trigger cellular adaptation processes.

Mitochondria contain two distinct aqueous subcompartments, the intermembrane space (IMS) and the matrix. Both subcompartments differ strongly with respect to their biological activity, their protein composition (Herrmann and Riemer, 2010) as well as their redox properties. Firstly, whereas the IMS is connected to the cytosol via porins, which allow the free diffusion of molecules of up to 5 kDa (including GSH/GSSG, NADPH/$NADP^+$ or hydrogen peroxide), the matrix is strictly separated from the IMS as the transport across the inner membrane is tightly controlled by substrate-specific carriers. Secondly, most cysteine residues of matrix proteins are believed to be reduced in the matrix, while many IMS proteins contain structural disulfide bonds which are introduced by the mitochondrial disulfide relay (also called MIA pathway) (Chacinska et al., 2004; Naoe et al., 2004; Allen et al., 2005; Mesecke et al., 2005; Bihlmaier et al., 2007; Banci et al., 2009; Kawano et al., 2009; Milenkovic et al., 2009; Bien et al., 2010; von der Malsburg et al., 2011; Fischer et al., 2013; Koch and Schmid, 2014).

The IMS may even be considered as two separate subcompartments because the peripheral IMS, which is adjacent to the outer membrane, is separated from the cristae space by cristae junctions (Frey and Mannella, 2000; Scorrano et al., 2002). Recently, a protein complex termed MICOS was identified and found to be located in the inner membrane and is critical for the separation of these two subcompartments as well as for mitochondrial functionality (Harner et al., 2011; Hoppins et al.,

2011; von der Malsburg et al., 2011; Körner et al., 2012). Cristae junctions restrict (and perhaps even control) the diffusion of molecules into and out of the cristae. Hence, the small molecule environment of the cristae space – specifically with regards to its pH, glutathione redox potential and levels of reactive oxygen species (ROS) – likely differs from that of the peripheral IMS and is more strongly influenced by the respiratory chain, which is mainly localized in cristae membranes. There is good evidence that proteins of the IMS and the inner membrane are partitioned between the cristae and the peripheral inner membrane but the mechanisms by which segregation takes place are unknown (Vogel et al., 2006; Wurm and Jakobs, 2006; Stoldt et al., 2012; Daum et al., 2013).

The mitochondrial matrix is separated from the IMS by the inner membrane, which only allows a tightly regulated exchange of small molecules. Thus, the small molecule environment of the matrix is mainly controlled independently from the remainder of the cell. This is nicely illustrated for example by the regulation of GSH/GSSG ratio in the matrix (Hu et al., 2008; Kojer et al., 2012). The matrix GSH/GSSG ratio is similar to its cytosolic counterpart, but is subject to completely different regulatory machineries including mitochondria-specific ROS-generating pathways (see below) and a partly mitochondria-specific set of reducing enzymes, including factors for the reductive regeneration of NADPH and GSH. The differences between the mitochondrial compartments lead to differences in the generation, handling and perception of oxidizing and reductive influences.

In the following we will illustrate this notion by describing and discussing the mechanisms that drive the reversible operation of molecular cysteine switches. Molecular switch operating is driven by the balance of opposing influences (Figure 1). Oxidizing influences mainly stem from the production of ROS at different sites in mitochondria; reducing influences ultimately originate from NADPH, which is regenerated by different metabolic pathways. Both thiol switch drivers rely on enzymatic machineries for the modulation and the relay of signals.

Redox influences and redox enzymes in mitochondria

Oxidizing influences in mitochondria

Endogenous ROS have for a long time been considered as the unwanted but unavoidable downside of mitochondrial metabolism that, over time, cause deleterious oxidative stress in eukaryotic cells. However, this traditional concept has been challenged by many recent studies which demonstrated that ROS (in particular hydrogen peroxide) also serve as critical signaling molecules (Chandel et al., 2000; Albrecht et al., 2011; Zarse et al., 2012; Mouchiroud et al., 2013; Gladyshev, 2014; Sies, 2014; Yee et al., 2014).

ROS encompass a variety of different molecules including the superoxide radical ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($^{\bullet}OH$) that are derived from the partial reduction of molecular oxygen (O_2). ROS can also result from the 'detoxification' of other ROS, as is the case during the dismutation of $O_2^{\bullet-}$ to H_2O_2 (and oxygen). H_2O_2 is a particularly suitable candidate to act as a signaling molecule, because of its relatively long half-life (Sies, 1993), relatively high concentration (nanomolar to low micromolar), and reasonable membrane permeability. In mitochondria channels in the outer membrane (porins) and the inner membrane, mitochondrial aquaporin-8 might considerably accelerate the exchange of hydrogen peroxide (Calamita et al., 2005; Marchissio et al., 2012). Perhaps most importantly, H_2O_2 can selectively oxidize specific cysteine residues in target proteins, making it an ideal candidate as a signaling molecule (Winterbourn and Hampton, 2008). H_2O_2 can oxidize thiols to sulfenic, sulfinic and sulfonic acid, of which the first two can be enzymatically converted back to thiols. Sulfenic acid can react with another thiol to a disulfide. In contrast, $^{\bullet}OH$ is highly reactive (reacting with diffusion-limited rate constants) and will indiscriminately oxidize biomolecules, rendering it unsuitable as a signaling molecule.

Mitochondrial pathways are major sources of cellular H_2O_2 (Figure 2). Different complexes of the respiratory chain release $O_2^{\bullet-}$ towards both the matrix and the IMS (Murphy, 2009; Bleier et al., 2014; Dröse et al., 2014). $O_2^{\bullet-}$ is rapidly converted to H_2O_2 by superoxide dismutases (copper-zinc SOD in the IMS and cytosol, manganese SOD in the matrix). The ~10 000 fold increase in the rate of $O_2^{\bullet-}$ dismutation by SODs not only accelerates the production of H_2O_2, but also competes with the production of peroxinitrite ($ONOO^-$) by the reaction of $O_2^{\bullet-}$ with $NO^{\bullet}$. While dedicated NO synthases have been reported in mammalian mitochondria (Giulivi et al., 1998; Nisoli et al., 2003), reports of a dedicated NO synthase in plant mitochondria have turned out to be incorrect (Gas et al., 2009); instead a likely source of mitochondrial NO is the mitochondrial respiratory chain by nitrite reduction (Gupta et al., 2011). Both H_2O_2 and $ONOO^-$ represent major electron sinks for the matrix thiol machinery and are likely to be main drivers behind thiol oxidation, both directly and indirectly.

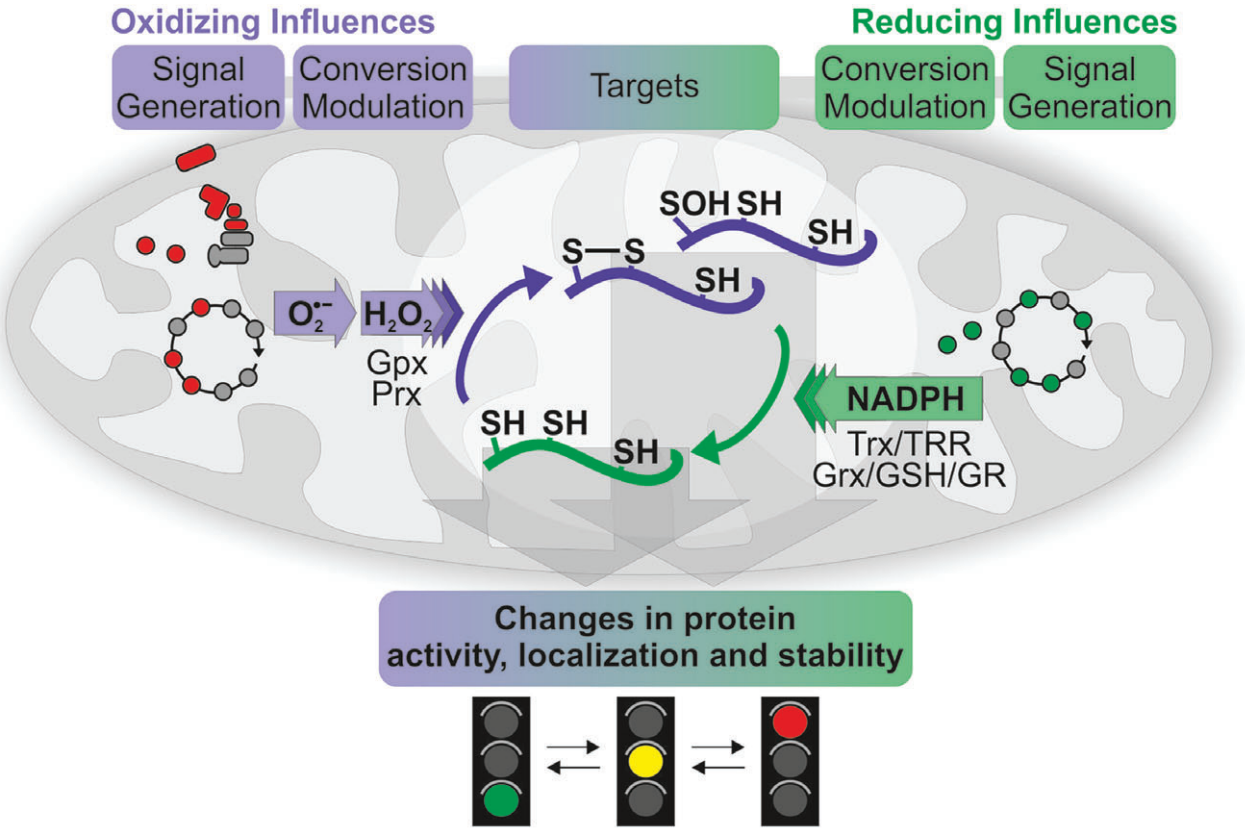

Figure 1: H_2O_2-controlled thiol switches in mitochondria.
Reactive oxygen species (ROS) can be produced at numerous sites in mitochondria, e.g. by flavine-containing enzymes of the TCA cycle or complexes of the respiratory chain. The main species produced is superoxide ($O_2^{\bullet-}$) that rapidly dismutates – either enzymatically or non-enzymatically – to hydrogen peroxide (H_2O_2). H_2O_2 reacts with its targets, which include reactive protein thiols or dedicated redox enzymes such as glutathione peroxidases (Gpx) or peroxiredoxins (Prx). The reaction with Gpx and Prx either leads to the 'detoxification' of H_2O_2 or can result in the indirect oxidation of proteins with less reactive thiols. The localization of the ROS source and the competition between degrading pathways and H_2O_2 diffusion determines which targets are oxidized. Thiol oxidation in target proteins can have different consequences like changes in protein activity, localization and stability (visualized by the traffic lights). Importantly, oxidation is counteracted by reducing systems that depend on the availability of NADPH and the activity of reducing systems [thioredoxin-thioredoxin reductase (Trx, TRR) and glutaredoxin, glutathione, glutathione reductase (Grx, GSH, GR)]. The SH shown in the figure depicts thiols which, *in vivo*, might be in their protonated or deprotonated state.

H_2O_2 can also be produced by flavin- or ubiquinone-utilizing enzymes and by the reaction of iron-sulfur clusters with $O_2^{\bullet-}$. The contribution of these different sources to mitochondrial H_2O_2 release in intact cells is unknown. The amounts of H_2O_2 produced and released by mitochondria may strongly fluctuate depending on the metabolic load (i.e. electron donors), the activity state of metabolic enzymes and the respiratory chain, the oxygen concentration, and the repertoire of redox enzymes. For instance, while the respiratory chain is commonly viewed as the main generator of $O_2^{\bullet-}$, isolated mitochondria have recently been reported to produce $O_2^{\bullet-}/H_2O_2$ at much higher rates from 2-oxoacid dehydrogenase complexes than from complex I under certain conditions (Quinlan et al., 2014; Goncalves et al., 2015).

ROS handling and reductive influences in mitochondria

ROS are subject to different enzymatic pathways that counteract or mediate (see below) their oxidizing influences (Figure 3). $O_2^{\bullet-}$ becomes rapidly dismutated by superoxide dismutases (SODs) in the IMS and the matrix (Sturtz et al., 2001). The product of this reaction – H_2O_2 – is subject to catalases, peroxiredoxins (Prx), glutathione peroxidases (GPx) and ascorbate peroxidases (APXs), which exist in distinct sets in the matrix and IMS. In the mitochondrial matrix, the contribution of catalase to H_2O_2 scavenging is limited (or even not existent) unless catalase is artificially overexpressed (Schriner et al., 2005). Prxs and GPxs become oxidized by the reduction of H_2O_2

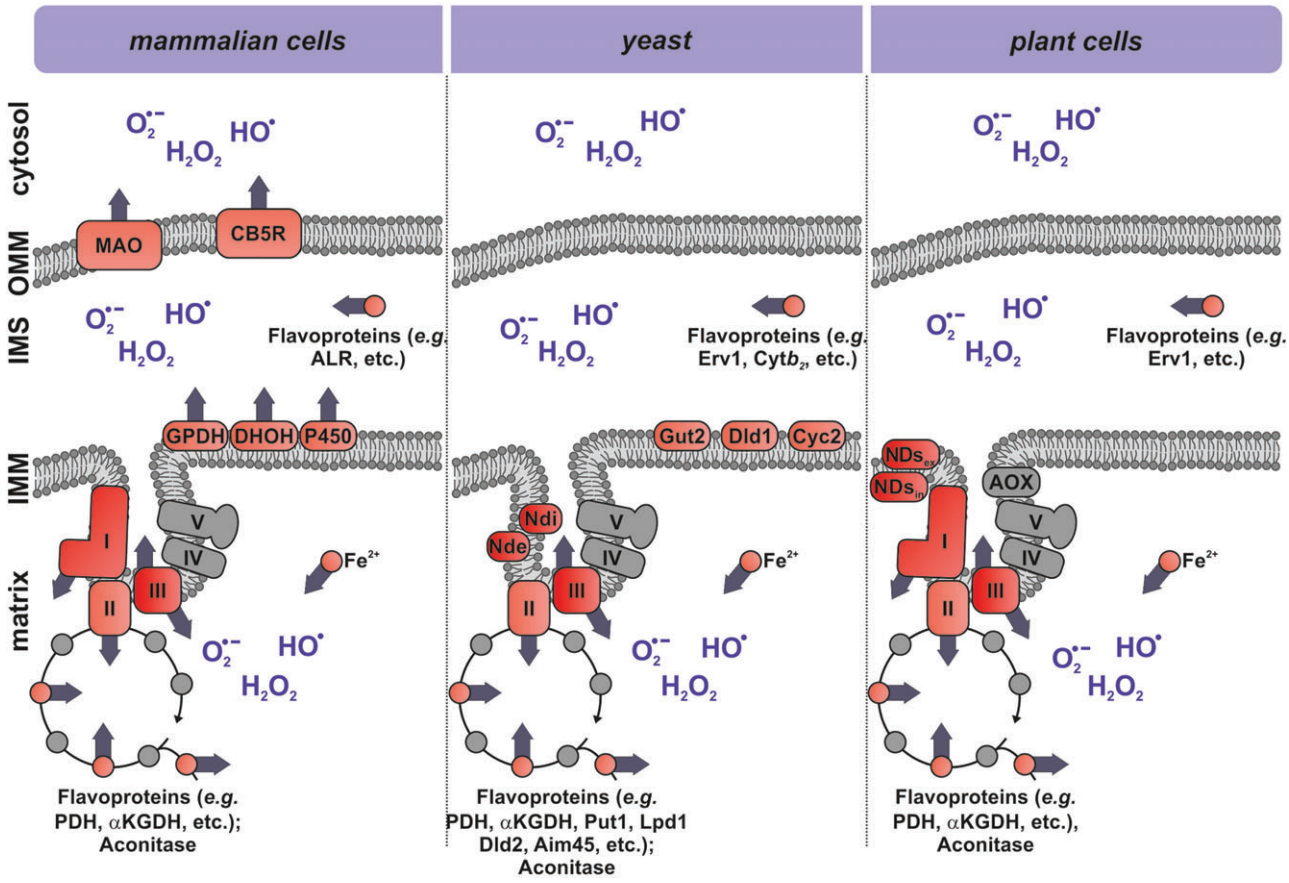

Figure 2: Sources of mitochondrial reactive oxygen species.
There are several sites in mitochondria that can, in principle, generate superoxide ($O_2^{\bullet-}$). The rates of superoxide production can vary with the local oxygen tension, potentials and half-life of redox centers, metabolic flux topology and rates, and the accumulation of damage in proteins. The site of production is of central importance for the physiological impact as it determines which ROS-handling systems and which targets the respective ROS will encounter. Sites for superoxide production include the complexes of the respiratory chain, which release superoxide towards the IMS (complex III) or matrix (complexes I, II and III), respectively. Moreover, flavoproteins in the different compartments may release ROS as byproducts of their catalytic activities. Players that may act as ROS sources because of their cofactor biochemistry are marked in red. Purple arrows show examples for which the generation of ROS was experimentally shown. αKGDH, α-ketoglutarate dehydrogenase; ALR, augmenter of liver regeneration; AOX, alternative oxidase; CB5R, cytochrome b_5 reductase; cytb$_2$, cytochrome *b*$_2$; DHOH, dihydroorotate dehydrogenase; GPDH, glycerol-3P-dehydrogenase; MAO, monoamine oxiadase; P450, cytochrome P450; PDH, pyruvate dehydrogenase

and have to be reduced by the thioredoxin (Trx) and glutathione/glutaredoxin (Grx) systems, respectively (Grant, 2001; Trotter and Grant, 2005; Kumar et al., 2011; Toledano et al., 2013). Prxs and GPxs are very fast enzymes (e.g. the cytosolic Prx2 catalyzes with a rate of 2×10^7 M^{-1}s^{-1}), and thus H_2O_2 reacts with them several orders of magnitudes more rapidly than with other protein thiols (Winterbourn and Hampton, 2008; Brandes et al., 2011). GPxs, and in particular GPx4 in mammals, also participate in the turnover of lipid hydroperoxides to their corresponding alcohols. Besides its documented reducing activity towards H_2O_2, human Prx5 is also known to reduce organic peroxides as well as peroxynitrite with second order rate constants of 10^6–10^7 M^{-1}s^{-1} whereas its reaction with hydrogen peroxide is lower (10^5 M^{-1}s^{-1}) (Dubuisson et al., 2004; Knoops et al., 2011). The solely matrix-located Prx3 shows second order rate constants with H_2O_2 of approx. 10^7 M^{-1}s^{-1} (Parsonage et al., 2008; Cox et al., 2009).

In plants ascorbate has been well characterized as part of the ascorbate-glutathione cycle (Halliwell-Asada cycle). In the plant mitochondrial matrix this cycle encompasses the enzymes glutathione reductase 2 (GR2), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR) and ascorbate peroxidase (APX) (Jimenez et al., 1997; Chew et al., 2003). DHAR draws electrons from GSH and MDHAR from NADPH to maintain a reduced ascorbate pool, which itself serves as electron donor for peroxide reduction via APX. Likewise electrons

for Prx and GPx reduction are eventually provided by NADPH either through thioredoxins (Trx) and thioredoxin reductase (Trr), or glutaredoxins (Grx) and glutathione reductase (Glr). Despite the differences among the reducing systems in mitochondria of animals, fungi and plants, in all cases the rapid production of NADPH from different metabolic pathways is key for the efficient functioning of the reductive pathways (Figure 3). NADPH may be produced by different pathways including NADP-dependent isocitrate dehydrogenase, malic enzyme and methylenetetrahydrofolate dehydrogenase. In addition NADPH can be produced in the matrix harnessing the NADH pool, either through transhydrogenases or though NADH kinases, such as Pos5 in yeast. The individual contribution of each pathway under physiological conditions is not clear (Outten and Culotta, 2003; Fan et al., 2014).

While the repertoires of antioxidative enzymes of the mitochondrial matrix and the cytosol are largely known and characterized (Figure 3), the set of antioxidative enzymes of the IMS remains poorly defined. Recent proteomics studies of the yeast IMS identified a thioredoxin (Trx1), thioredoxin reductase (Trr1), the glutathione

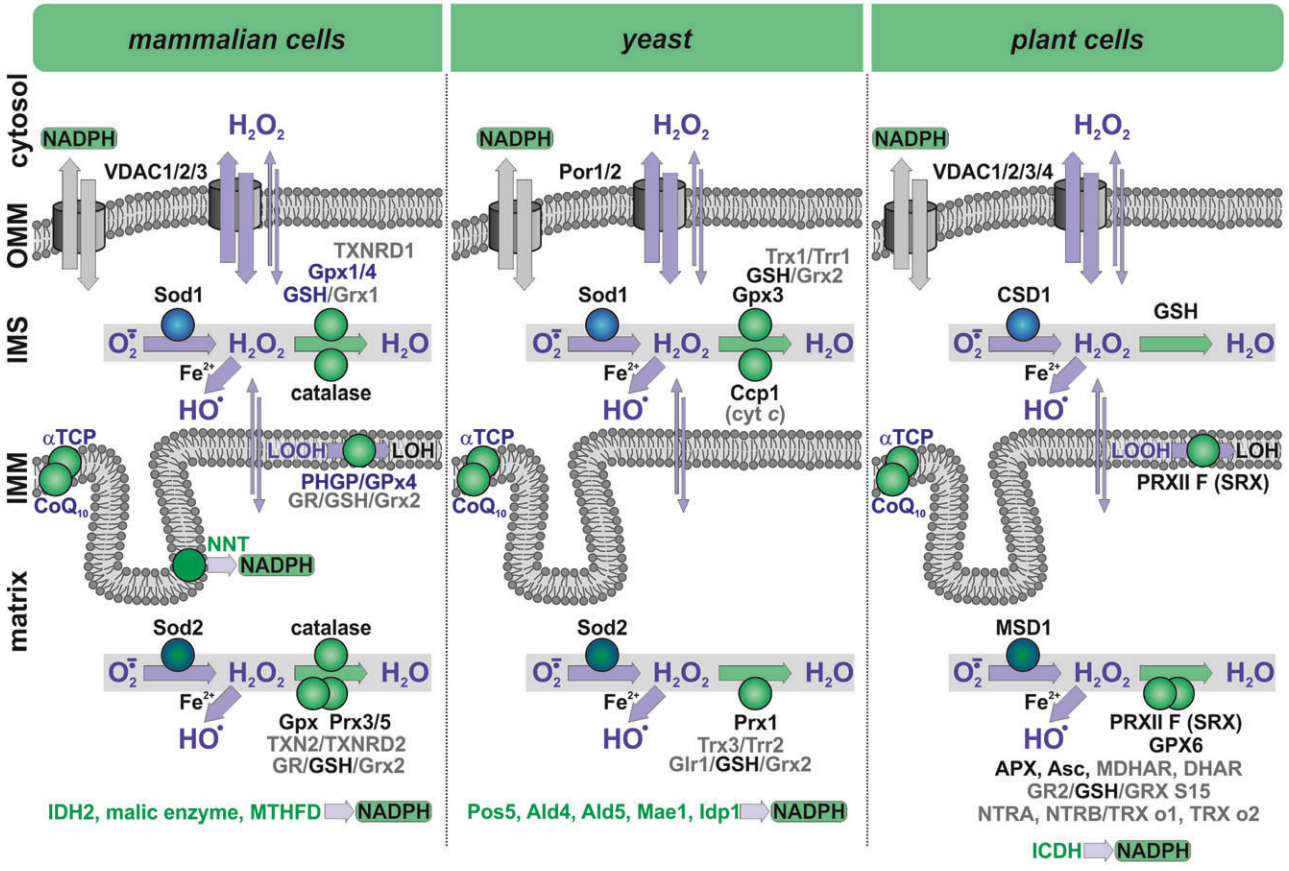

Figure 3: Enzymatic and non-enzymatic systems for the conversion and degradation of mitochondrial reactive oxygen species. Superoxide ($O_2^{\bullet-}$) as main ROS generated *de novo* is rapidly dismutated to H_2O_2, either non-enzymatically or by superoxide dimutases (SODs), which accelerate this reaction by a factor of about 10 000. H_2O_2 can leave the respective compartment by diffusing through membranes or in the case of the outer membrane by diffusing through porins/voltage-dependent anion channels (VDAC). This might play an important role during ROS signaling from mitochondria to the remainder of the cell. H_2O_2 can also undergo Fenton chemistry catalyzed by Fe^{2+} resulting in the generation of highly reactive hydroxyl radicals ($^{\bullet}OH$). However, the majority of H_2O_2 is presumably scavenged by multiple enzymatic and non-enzymatic systems, which convert H_2O_2 to water. These systems strongly differ between compartments and compete with each other and other proteins in the same compartment for reaction with H_2O_2. As electron donor these systems rely on NADPH, highlighting the importance of systems for NADPH regeneration *in situ*. APX, ascorbate peroxidase; αTCP, α-tocopherol; Ccp, cytochrome *c* peroxidase; CoQ_{10}, coenzyme Q_{10}; DHAR, dehydroascorbate reductase; Glr/GR, glutathione reductase; GPX/GPx/Gpx, glutathione peroxidase; GRX/Grx, glutaredoxin; GSH, glutathione; IDH/ICDH, isocitrate dehydrogenase; MDHAR, monodehydroascorbate reductase; NNT, nicotin nucleotid transhydrogenase; NTR/Trr/TXNRD, NADPH-thioredoxin reductase; PHGP, phospholipid hydroperoxide glutathione peroxidase; Por, porin; Prx/PRX, peroxiredoxin; Sod1/CSD1, copper-zinc superoxide dismutase; Sod2/MSD1, manganese superoxide dismutase; SRX, sulfiredoxin; TRX/Trx/TXN, thioredoxin; VDAC, voltage-dependent anion channel

peroxidase Gpx3, cytochrome *c* peroxidase (Ccp1) and the copper/zinc SOD (Sod1) (Vögtle et al., 2012). In addition, we have identified Grx1 and Grx2 activity in the IMS of yeast cells (Kojer et al., 2014). In humans, Grx1, Sod1, TXNRD1 (Inarrea et al., 2007), GPx1 and GPx4 (Liang et al., 2009) were found to localize to the IMS (Pai et al., 2007). Even more incomplete is our knowledge on the plant IMS. The presence of a copper/zinc SOD (Cu/ZnSOD1, CSD1) has been suggested, but remains to be unambiguously shown (Huang et al., 2012).

Small redox molecules like alpha-tocopherol (vitamin E) and ubiquinone (coenzyme Q) support redox enzymes in their role in ROS depletion. These compounds are localized to the membranes and serve as scavengers of lipid peroxyl radicals. Therapeutic strategies using small molecules to reduce mitochondrial ROS levels are currently under intense development and testing (Smith et al., 2012).

In this context it is also important to note that H_2O_2 can (slowly) diffuse across membranes or (much faster) through porins/voltage-dependent anion channels (VDACs) in the outer membrane and presumably through aquaporins in the inner membrane. Hence, mitochondrial H_2O_2 has great potential to also influence reactions outside of mitochondria whereas the relevance for other mitochondria-generated ROS might be largely confined to the organelle.

Physiological role of ROS-handling enzymes in mammals

The importance of maintaining mitochondrial ROS at physiological levels has been demonstrated by specifically deleting selected mitochondrial redox enzymes in mice (Table 1). For instance, mice with targeted deficiencies in some members of the matrix-located thioredoxin-2/thioredoxin reductase-2/peroxiredoxin III axis and SOD2 develop severe phenotypes. The respective phenotypes include aberrations in embryonic brain, heart and blood cell development and neurodegeneration. In contrast, Grx2 deficient mice present more specific defects affecting lens epithelial cells (Wu et al., 2011), as well as cardiac tissue and skeletal muscle. These effects are probably a consequence of increased proton leakage and perturbed oxidative phosphorylation caused by dysregulation of glutathionylation and de-glutathionylation events in the electron transport chain complexes (Mailloux et al., 2013, 2014). For enzymes of the IMS, it appears to be more challenging to unequivocally assign distinct functions for redox enzymes in the control of ROS levels in the IMS as some of these, including SOD1, Grx1, TXNRD1, Gpx1 and Gpx4, are known to dually localize to the cytosol, IMS and other compartments of the cell (Table 1). Therefore, phenotypes and mechanisms obtained by reverse genetic studies in mice and cells need to be carefully interpreted as the contribution of the different compartments to a given phenotype can often not easily be attributed to these enzymes. This is, for instance, nicely illustrated for the short form of mammalian Gpx4 (also referred to as the 'cytosolic' form) that is present in the cytosol, nucleus and in the IMS, where it was found to be strongly associated with the outer leaflet of the inner membrane of mitochondria (Liang et al., 2009). As Gpx4 is efficiently reducing oxidized lipids in lipid bilayers, it is intriguing that cardiolipin, a phospholipid specific for the inner membrane of mitochondria, was found to be the first phospholipid to be oxidized in kidneys of inducible GPx4 knockout mice (Friedmann Angeli et al., 2014). The physiological role of Prdx6 is far from being clear as it fails to compensate for Gpx4 loss. This might be due to its dual function as Prdx6 does not directly act on oxidized esterified lipids and requires the release of fatty acid through its phospholipase A_2 (PLA_2) activity. Hence, Gpx4 and Prdx6 work via two different mechanisms as membrane repair via Prdx6 will cause a substantial loss of fatty acids from lipid molecules and thereby substantially impacts on membrane structure because of an increase of lysophospholipids (these are known to be strong detergents) in membranes. In contrast, Gpx4 acts directly on oxidized esterified fatty acids, thereby reducing them to the corresponding alcohols in membranes.

Despite the myriad of reported phenotypes in enzymes contributing to mitochondrial ROS control (Table 1), it is still astonishing that almost nothing is known about the *in vivo* mechanisms of specific thiol switches in mitochondrial processes, including cell death signaling and bioenergetics that might be mediated by some of these enzymes in mammals.

Specific mitochondrial proteins are prone to undergo cysteine oxidation

Numerous *in vitro* studies have reported on the sensitivity of specific enzymes towards oxidants. The physiological relevance of these studies is often not clear as oxidative damage will arguably compromise the activity of any protein if sufficiently high levels of oxidants are applied. In the majority of cases studied, the inhibitory concentrations, for example of H_2O_2, are considerably larger than the concentrations found *in vivo* (Cocheme et al., 2011). Nonetheless, several recent proteomic analyses have reported oxidative (thiol) modifications on a number of

Table 1: Summary of knockout mice for redox regulating enzymes with mitochondrial localization.

Gene	Localization	Major pathophysiologic phenotype(s)	References
SOD1	Cytosol IMS	Increased susceptibility to ischemic and toxic insults; age-dependent skeletal muscle atrophy; glucose intolerance; altered bioenergetic function in mice	Huang et al. (1997); Muller et al. (2006); Muscogiuri et al. (2013); Garratt et al. (2014)
SOD2	Matrix	Neurodegeneration and early postnatal death of mice; dilated cardiomyopathy; increased sensitivity of hemizygous mice to various stress-inducing agents and accelerated aging	Li et al. (1995); Lebovitz et al. (1996); Flynn and Melov (2013)
Grx1	Cytosol IMS	Impaired NF-κB signaling; increased susceptibility to UVR-induced lens injury; attenuated actin polymerization and consequently impaired recruitment of neutrophils to inflammation sites and reduced bacterial handling	Reynaert et al. (2006); Kronschläger et al. (2012); Sakai et al. (2012)
Grx2	Matrix	Increased sensitivity to oxidative stress in primary mouse lens epithelial cells; increased levels of glutathionylated proteins and decreased oxidative phosphorylation in cardiac and skeletal muscle	Wu et al. (2011); Mailloux et al. (2013, 2014)
GPx1	Cytosol IMS	Increased susceptibility to ischemic and toxic insults; increased atherosclerosis; increased insulin sensitivity	Crack et al. (2001, 2006); Lewis et al. (2007); Wong et al. (2008); Loh et al. (2009)
GPx4	Cytosol Nucleus Plasma membrane IMS	Early embryonic lethality; neurodegeneration in different brain regions; aberrant hair follicle development; increased thrombus formation in vitamin E-deprived endothelial-specific Gpx4 null mice; acute renal failure	Yant et al. (2003); Seiler et al. (2008); Wirth et al. (2010); Sengupta et al. (2013); Wortmann et al. (2013); Friedmann Angeli et al. (2014)
TXNRD1	Cytosol IMS	Widespread developmental retardation and embryonic death between E8.5 and E10.5; cerebellar hypoplasia; increased resistance of liver to acetaminophen poisoning	Jakupoglu et al. (2005); Soerensen et al. (2008); Patterson et al. (2013)
TXN2	Matrix	Embryonic death at E10.5 and increased apoptosis rates in the developing brain	Nonn et al. (2003)
TXNRD2	Matrix	Embryonic death hat E13.5 caused by impaired development of fetal blood cells and cardiac tissue; impaired cardiac function in response to transient ischemia	Conrad et al. (2004); Horstkotte et al. (2011)
PrxIII	Matrix	Increased susceptibility to LPS-induced oxidative stress and decreased survival of macrophages; impaired synaptic plasticity	Li et al. (2007, 2009)

mitochondrial enzymes, although in most cases it remains unclear which proportion of a given protein is modified and whether these modifications really influence mitochondrial pathways.

Redox regulation of the respiratory chain

The addition of H_2O_2 to cells was reported to preferentially damage (or modify) mitochondrial proteins (Garcia et al., 2010; Perluigi et al., 2010; Qin et al., 2011; Martinez-Acedo et al., 2012; Stauch et al., 2014). In several investigations H_2O_2 addition reduced respiratory activity and mitochondrial membrane potential. This might point at feedback control which limits electron flow in the respiratory chain under oxidative conditions in order to reduce the production of additional $O_2^{\bullet-}$ and, hence, H_2O_2. The way in which ROS interact with the respiratory chain is apparently highly complex and the numerous publications on the redox regulation of respiration have identified different enzymes of the electron transport chain as the primary responders to oxidation. A recent review provides a good summary of our current understanding of thiol-based redox regulation of the respiratory chain (Dröse et al., 2014).

Complex I (NADH ubiquinone oxidoreductase) has recently been found to feature a molecularly well-defined thiol redox switch that appears to be able to control respiration and superoxide production *in vitro*

and *in vivo* (Chouchani et al., 2013). Cysteine residue 39 of the mammalian ND3 subunit of the complex, which is in proximity to the quinone binding site, is particularly susceptible to thiol modification under low oxygen conditions such as after ischemia in myocardial infarction and stroke (Galkin et al., 2008; Babot et al., 2014). Upon re-oxygenation (reperfusion) rapid reactivation of complex I is a major source of the deleterious ROS production in cardiomyocytes (Yellon and Hausenloy, 2007; Prime et al., 2009; Chouchani et al., 2014). Transient inactivation of complex I in these patients thus holds potential as a powerful therapeutic strategy. Hence, modification of this thiol in complex I (for example by nitrosylation) prevents reactivation of complex I and therefore protects against ischemia-reperfusion injury. The recently solved crystal structures of complex I (Hunte et al., 2010; Vinothkumar et al., 2014) will provide an excellent basis to unravel the molecular mechanisms by which this molecular switch on ND3 is controlled. It appears likely that this switch is also used under physiological conditions to prevent the burst of respiration upon rapid changes in the prevalent oxygen concentration of tissues. Conservation of the critical cysteine of ND3 across different eukaryotic kingdoms, such as animals and plants, is indicative of a fundamental and physiological role in regulating mitochondrial respiration beyond specific pathologies or stress conditions.

Redox regulation of mitochondrial enzymes with catalytic cysteine residues

A recent proteome-wide study identified oxidation-prone cysteine residues in mouse heart cells on the basis of their reactivity towards an iodoacetamide-containing compound (Weerapana et al., 2010). Interestingly, among the 50 most reactive cysteine residues in the cell, 19 are found in mitochondrial proteins. Proteins with such hyper-reactive cysteine residues include 'professional' redox enzymes such as thioredoxin Trx2 (also called Txn2) or the matrix superoxide dismutase Sod2. However, by far most mitochondrial proteins with hyper-reactive cysteine residues are metabolic enzymes, and the reactive cysteines are very often, but not always, critical residues in the reactive center. Examples of enzymes that may contain physiologically relevant thiol switches will be introduced in the following.

Aldehyde dehydrogenases

The aldehyde dehydrogenase Aldh2 was listed as the protein with the most reactive cysteine residue in mitochondria (Weerapana et al., 2010). Other aldehyde dehydrogenases were identified in the same screen, including Aldh5 and Aldh6. Aldehyde dehydrogenases, and in particular Aldh2, have been known to contain redox-sensitive thiol groups that are subject to oxidative inactivation (Loomes and Kitson, 1989; Moon et al., 2005; Wang et al., 2011) (Figure 4A). Recent studies on the reaction mechanism of aldehyde dehydrogenases indicated a transient covalent bond of the conserved reactive cysteine residue with the nicotinamide ring of $NADP^+$ (Diaz-Sanchez et al., 2011; Tsybovsky et al., 2013).

Aldehyde dehydrogenases are critical for the detoxification of aldehydes, including those derived from lipid peroxidation. They are of high medical relevance as their inactivation causes alcohol-induced cell damage and cardiotoxicity (Chen et al., 2010). Polymorphisms in aldehyde dehydrogenases may explain why some alcoholism patients acquire certain organ-specific complications whereas others acquire different ones (Chao et al., 1997). Oxidative inactivation of Aldh2 is reversed by addition of dithiotreitol (DTT), which presumably resolves an inactivating disulfide in the active site (Wenzel et al., 2007). It is unclear whether the extreme redox-sensitivity of aldehyde dehydrogenases is the unavoidable consequence of their mode of action or whether it serves a purpose under physiological (or pathological) conditions to repress enzyme activity upon oxidative stress.

Mitochondrial thiolases

The mitochondrial matrix contains a number of thiolases (also known as acetyl-coenzyme A acetyltransferases) for the catabolic breakdown of fatty acids and other coenzyme A-activated metabolites. Cysteine residues of several mitochondrial thiolases such as acetoacetyl-CoA thiolase, ketoacyl-CoA thiolase and the 2-ketoacyl-CoA thiolase are among the most reactive protein thiols in mammalian cells (Weerapana et al., 2010). These enzymes are characterized by reactive cysteine residues in their active centers that form acyl-thioester reaction intermediates with their substrates (Modis and Wierenga, 1999; Kim and Battaile, 2002; Haapalainen et al., 2006). A number of recent studies suggest that oxidative modifications of these reactive cysteines are linked to diseases. For example the activity of the mitochondrial acetoacetyl-CoA thiolase was found to be reduced by 80% in colon cells of patients suffering from ulcerative colitis because of increased mitochondrial H_2O_2 levels (Santhanam et al., 2007). Reduced thiolase activity in ulcerative colitis was returned to normal by exposure to reductants (Santhanam et al., 2007). Redox-induced

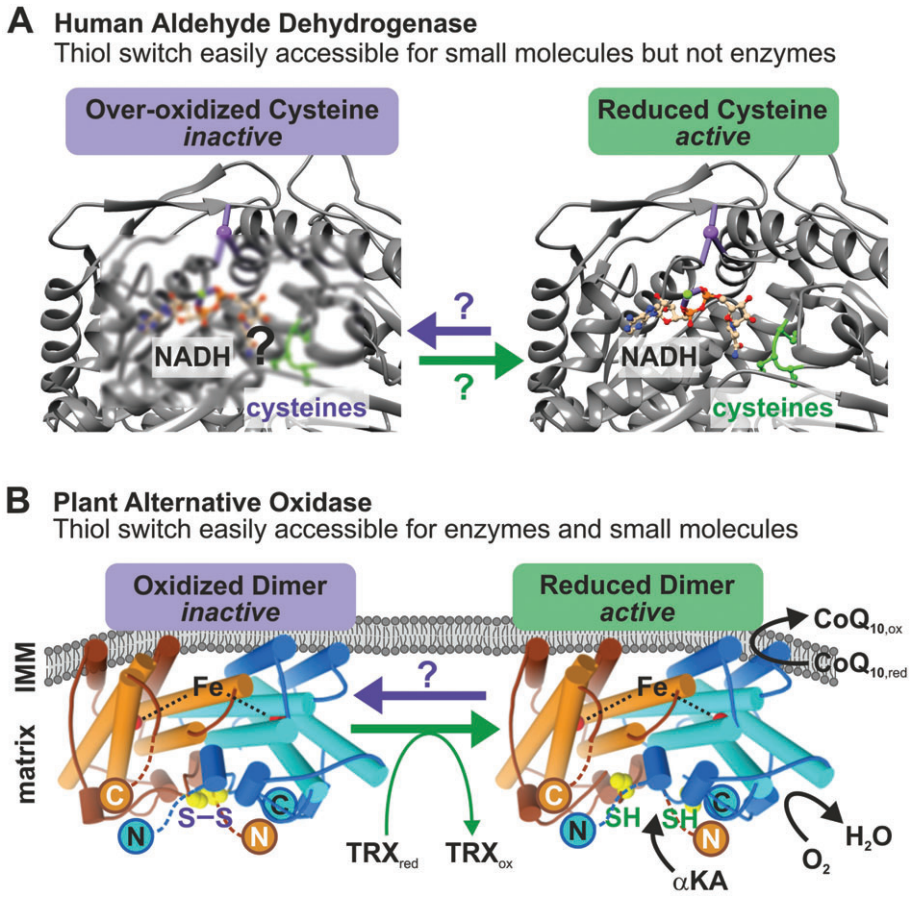

Figure 4: Examples of proteins with potential thiol switches.
(A) Structure of the active site of the human aldehyde dehydrogenase. The three adjacent cysteine residues are shown in green and purple, representing their reduced and oxidized states, respectively. They are close to the bound NADPH cofactors and it was suggested that one of them forms a covalent reaction intermediate with this cofactor during the reaction cycle. Oxidation of this cysteine would consequently abolish enzymatic activity. As the cysteine is deeply buried in the active center of the enzyme, it is likely that the oxidized form of the cysteine is stabilized because of it is partially inaccessible to reducing redox enzymes. Shown is the structure of chain A from PDB 1O02 (Perez-Miller and Hurley, 2003). (B) Potential thiol switch in plant alternative oxidase (AOX). AOX forms homodimers, which form an intermolecular disulfide bond *in vitro* (shown in green and purple). Reduction, which can be mediated by thioredoxins (TRX), opens the disulfide priming the dimer for activation by α-ketoacids (αKA). In the active form reduced coenzyme Q10 [CoQ_{10}(red)] from the electron transport chain is oxidized [CoQ_{10}(ox)] and its electrons are passed to oxygen, which is eventually reduced to water by the active sites. The structure model shown has been generated based on the crystal structure of *Trypanosoma brucei* AOX (PDB 3VV9) (Shiba et al., 2013) using the sequence of *Arabidopsis thaliana* AOX1a. The N-termini are highly flexible and *T. brucei* AOX does not contain the cysteine residue. This makes the depicted thiol-switch-dependant conformational change hypothetical.

inactivation of the acetoacetyl-CoA thiolase was also reported for other cells under pathological conditions, such as in hepatocytes suffering from alcohol-mediated mitochondrial dysfunction or in cardiomyocytes of chronically diabetic rats (Grinblat et al., 1986; Moon et al., 2006).

Creatine kinase

Creatine kinases are expressed in many animal cells, particularly in those with high energy demand such as myocytes or neurons. The mitochondrial isoform of creatine kinase, which is localized in the intermembrane space, uses ATP to phosphorylate creatine. The resulting phosphocreatine diffuses into the cytosol where it is used by the two cytosolic isoforms of creatine kinase to phosphorylate ADP. All three isoforms contain a highly conserved cysteine residue, which is important although not essential for activity (Okinaka et al., 1964). The cysteine residue of creatine kinase has a very low pK_a of 5.6 so that it is predominantly present in the reactive thiolate anion form (Wang et al., 2001, 2006). This reactive cysteine was shown to be sensitive to H_2O_2 or glutathione disulfide and could be reactivated by the addition of DTT or glutathione (Suzuki

et al., 1992; Thomas et al., 1994; Reddy et al., 2000; Wang et al., 2001). Overoxidation of creatine kinase was reported under pathological conditions for example in cancer or ischemic heart cells (Mekhfi et al., 1996; Choi et al., 2001). It is not known what percentage of the creatine kinase pool is oxidized *in situ*. However, as creatine kinase is rate-limiting for myocardial energetics (Gupta et al., 2013), oxidative inactivation may be very relevant *in vivo*.

Aconitase and homoaconitase

Aconitase catalyzes the conversion of citrate to isocitrate in the TCA cycle and contains an iron-sulfur cluster of the 4Fe-4S type in its active site. In contrast to 2Fe-2S clusters as found in ferredoxins, 4Fe-4S clusters are intrinsically labile and very sensitive towards oxidation. They also might lead to the generation of a hydrogen radical by a Fenton-like mechanism (Vasquez-Vivar et al., 2000). Numerous studies have reported the oxidative modification of aconitase and homoaconitase, which result in their inactivation during aging or in oxidative stress-related disorders (Bulteau et al., 2005). A number of recent articles indicated that the oxidative damage of aconitase might be of physiological relevance (Matasova and Popova, 2008; Lushchak et al., 2014; Stehling et al., 2014). In mammalian cells, there is a cytosolic isoform of aconitase which is also referred to as iron responsive protein that serves as transcription factor when it loses the 4Fe-4S cluster (Walden et al., 2006).

Studies on model organisms suggest that even under *in vivo* conditions considerable fractions of 4Fe-4S cluster-containing proteins carry cysteine modifications: in yeast 35% of homoaconitase was found to contain disulfides (Brandes et al., 2011). Similarly, 37% of aconitase was found to be thiolated in *Escherichia coli* under unstressed conditions and this number increased to 68% after treatment of cultures with hypochlorous acid (Leichert et al., 2008).

Branched chain aminotransferase

The catalytic breakdown of branched chain amino acids (isoleucine, leucine, valine) is catalyzed by mitochondrial enzymes. In the first step of this pathway, the amino acids are converted by the mitochondrial isoform of branched chain aminotransferase (BCATm) to their respective α-ketoacids. In mammals, including humans, BCATm contains a cysteine pair (C315 and C318 in the human enzyme) that forms a disulfide bond under oxidizing conditions (Conway et al., 2002, 2003). C315 has a low pK_a and is hence mainly present in a thiolate anion form that reacts efficiently with H_2O_2 to sulfenic acid and further to the disulfide (Conway et al., 2004). The same residue is also prone to nitrosylation by NO (Coles et al., 2009). Thiolation as well as nitrosylation thereby leads to the inactivation of the enzyme which is fully reversible upon reduction. Interestingly, this thiol switch is not present in closely related orthologs of nematodes, insects or bacteria, suggesting that it evolved in branch chain aminotransferases of mammals to allow redox-regulation of amino acid catabolism. A recent study proposed that BCATm can use its disulfide bond to oxidize other proteins in a reaction similar to that of the oxidoreductases Mia40 and protein disulfide isomerase and thus promote oxidative protein folding in the matrix (El Hindy et al., 2014). While this is an intriguing hypothesis, direct evidence for such a role under *in vivo* conditions in the mitochondrial matrix is still missing.

Alternative oxidase and other targets for thiol switching in plants

The respiratory chain of plant mitochondria contains an alternative oxidase (AOX) that passes electrons directly from ubiquinone to oxygen without pumping protons. AOX activity does not lead to ATP production (as no protons are pumped) and hence uncouples respiratory metabolism from energy conservation. The control of AOX activity is important to balance metabolic redox status and cellular energy charge, depending on nutrient status and environmental conditions of the plant. AOX is only fully active as a dimer in a reduced state, when it can be activated by α-keto acids, such as pyruvate (Millar et al., 1993) (Figure 4B). The requirement for reduction has been convincingly shown *in vitro* (Umbach and Siedow, 1993; Umbach et al., 1994, 2002; Rhoads et al., 1998) and can be overcome in mutants of the critical cysteine at position 78 (Cys-78). TRXs can catalyze the reduction *in vitro* (Gelhaye et al., 2004; Yoshida et al., 2013). As redox dependence of AOX activity has been predominantly studied in isolated mitochondria and protein thiols oxidize during mitochondrial isolation, the significance of this thiol switch under physiological conditions is still unclear. Overexpression of a Cys-78 mutant of AOX1a lacking the thiol switch in *Arabidopsis* does not show a phenotype as compared to over-expressors of the wild type form (although overexpression was not performed in the KO background meaning that a mix of wild type and mutant protein will be present)(Umbach et al., 2005). It has been argued that AOX may stably exist in its reduced state *in vivo* and that oxidation is merely an experimental

artefact. However, in some plants [young soybean roots (Millar et al., 1998); wheat leaves (Bartoli et al., 2005)] an oxidized population of AOX is found that is reduced, i.e. activated, depending on developmental state and stress impact (drought). In other systems no oxidized AOX population has been found. The means of analyzing AOX thiol redox status *in vivo* require improvement to decide what physiological significance this thiol switching may have for plant AOX.

Two further candidates for matrix proteins with thiol switches of plants were recently discovered: citrate synthase (Schmidtmann et al., 2014) and NAD-dependent isocitrate dehydrogenase (Yoshida and Hisabori, 2014). Both can be reduced in a thioredoxin-mediated manner *in vitro*, and the induced thiol switch can strongly alter enzymatic activity, opening the possibility of redox dependent regulation. Yet, it remains unclear whether those thiol switches can regulate metabolic flux *in vivo*.

ROS and the redox state of cysteine residues with low reactivity – the relevance of enzymes for specificity and kinetics

How can H_2O_2 oxidize cysteine residues in a specific, localized and reversible manner? H_2O_2 reacts only slowly with most protein thiols (Winterbourn and Hampton, 2008) while catalytic thiols of dedicated detoxifying enzymes (catalase, GPx, Prx, APX) make exceptions from that rule (Forman et al., 2014). Despite the high reactivity and abundance of these H_2O_2-detoxifying enzymes, a diverse set of proteins containing cysteine residues of low reactivity towards H_2O_2 appear to be targets for H_2O_2-induced oxidation. Different models that are not mutually exclusive have been put forward to explain how this could be explained mechanistically (Figure 5). All of them emphasize the importance of indirect, enzyme-mediated transfer of redox status from oxidants or reductants onto target proteins.

– *The enzyme-mediated oxidation model* Prx and GPx serve as mediators of H_2O_2 signaling. They react rapidly with H_2O_2 via their highly reactive thiols, which are oxidized in turn to subsequently transfer the oxidation to specific target molecules that otherwise have a low reactivity towards H_2O_2 directly. Given the dominating role of kinetic regulation in thiol redox biology, this type of regulation is likely to act as a major operator of physiological switches. One (non-mitochondrial) example for such a mode of action is the yeast transcription factor Yap1, which resides in the cytosol under non-stress conditions (Delaunay et al., 2002). Upon exposure of cells to

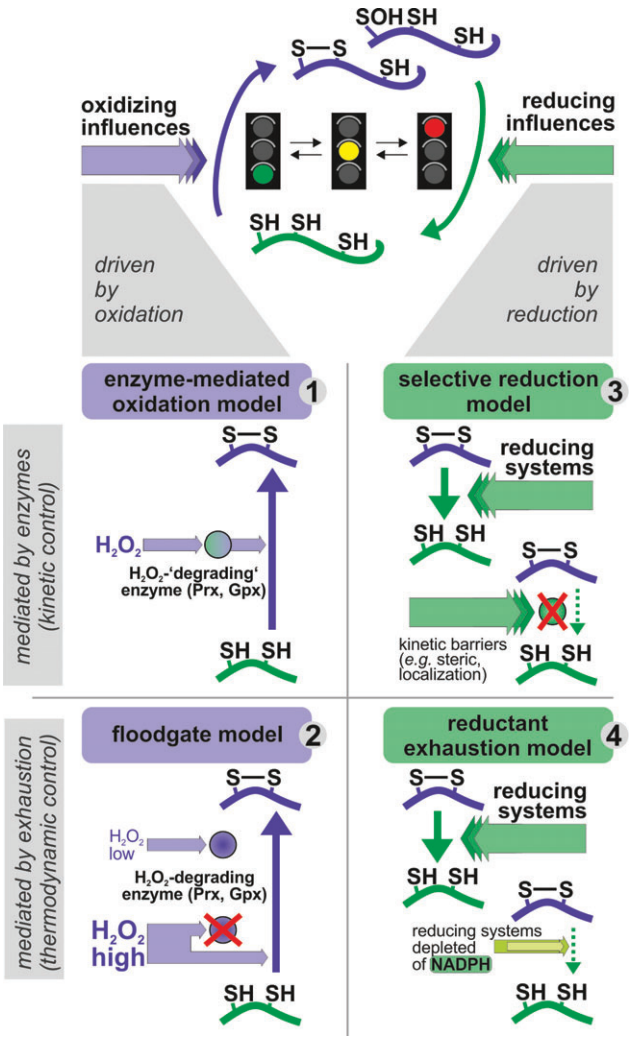

Figure 5: Concepts for operation and maintenance of mitochondrial protein thiol switches based on cysteine residues with low reactivity towards hydrogen peroxide.
The origins of redox perturbations that operate a mitochondrial protein thiol switch with low reactivity towards H_2O_2 are still not clearly resolved. As a basic requirement a thermodynamic drive to oxidize a reduced thiol switch or to reduce an oxidized one must exist, which is fulfilled in the mitochondrion by the presence of H_2O_2 and a sufficiently reducing NADPH pool, respectively. The balance between oxidizing and reducing pressures generates a functional steady-state of the thiol switches within a compartment. Different, distinct steady-states can be adopted by shifting the rate of oxidation and reduction, respectively. Changes can be controlled either kinetically [models (1), (3)] or thermodynamically [models (2), (4)], by modifying the oxidizing [models (1), (2)] or the reducing side [models (3), (4)]. This is illustrated by four models, which together provide a realistic concept for the operation and maintenance of protein thiol switches *in vivo*. See text for a detailed explanation of the individual models.

H_2O_2, Yap1 receives a disulfide bond from GPx3, which has itself been oxidized by H_2O_2. This mediates relocalization of Yap1 to the nucleus to elicit its function in transcriptional regulation.

- *The floodgate model* The generation of large amounts of H_2O_2 saturate (or inactivate by oxidation) the fast detoxifying pathways leaving slower-reacting thiol groups in target proteins time for reaction or allowing new functions for these 'over-oxidized' detoxifying enzymes (Wood et al., 2003; Kil et al., 2012). These more slowly reacting cysteine residues may either operate as thiol switches or fulfill a role as 'redox buffer' (Hansen et al., 2009; Brandes et al., 2011). In addition, micro-compartmentation and diffusion gradients may play a critical role: protein thiols with lower reactivity but localized in close proximity to the site of H_2O_2 generation may preferentially react with H_2O_2 when the more highly reactive H_2O_2 scavengers are localized far away. This model may, for instance, account for the mitochondrial IMS, which appears to contain only limited amounts of fast detoxifying systems (Kojer et al., 2014).
- The oxidative influences are balanced and fine-tuned by reducing pathways, leading to a dynamic equilibrium between oxidizing and reducing inputs to set the duration and extent of cysteine residue oxidation as a result. The significance of the reductive pathways in the dynamic regulation of protein thiol switches are summarized in the following models:
- *The selective reduction model* Despite sufficient thermodynamic drive for reduction, only specific cysteine residues are efficiently reduced by the Trx and Grx systems, because of steric, i.e. kinetic, constraints. This increases the extent and half-life of oxidation of non-target as compared to target cysteine residues, assuming equal rates of oxidation. A given cysteine residue may even switch between being a target and a non-target depending on structural re-organization of protein associations or complexes which alter the accessibility of the reduction machinery to the thiol switch.
- *The reductant exhaustion model* Under conditions of overwhelming oxidation or upon insufficient flux through the metabolic pathways reducing $NADP^+$, NADPH is depleted (oxidized, i.e. low NADPH/$NADP^+$ ratio causing less reducing redox potential) and the thermodynamic drive from reduction decreases. This will result in increased amounts of oxidized thiols for prolonged times. Such an 'NADPH depletion' scenario is unlikely to be useful for physiological regulation as it is not specific to a given thiol switch; instead mitochondrial thiol switches will be oxidized in the order of their reactivity and if NADPH depletion persists long enough in the order of their midpoint potential starting from the most reducing couples (most negative potentials). Nevertheless more general responses may be triggered by exhaustion of reductant, for instance in mitochondrial pathology or in the early stages of mitochondria-mediated cell death.

Importantly, both models of oxidation (1+2) ultimately result in the oxidation of cysteine residues, even those with a low reactivity towards H_2O_2. Disulfides, sulfenic acids or glutathionylated cysteine residues that are formed in turn may serve in translating the H_2O_2 signal into a change in protein activity, localization or stability. The reductive pathways (3+4) balance oxidation generating a dynamic equilibrium in which the extent and duration of cysteine residue oxidation is shaped by all inputs (models 1, 2, 3, 4).

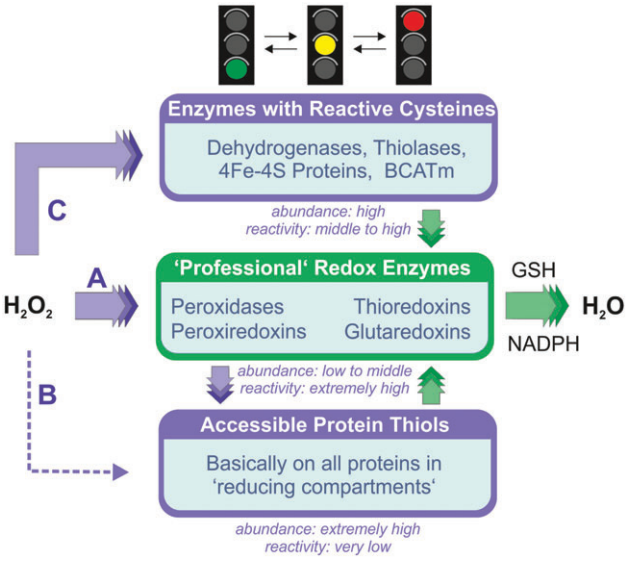

Figure 6: Enzymes with highly reactive cysteine residues might be direct targets of hydrogen peroxide.
(A) H_2O_2 efficiently reacts with professional redox enzymes such as glutathione peroxidases or peroxiredoxins. (B) Protein thiols can also directly react with H_2O_2 resulting in higher oxidation states, such as sulfenic acid. However, their very low reactivity largely prevents this reaction, which is therefore presumably only relevant under highly oxidizing conditions or if mediated by other enzymes. (C) In contrast, enzymes with highly reactive cysteine residues, which are abundant in mitochondria, are good candidates to serve as direct targets for regulation by H_2O_2. Their reduction depends on glutaredoxins and thioredoxins, but the rates of their recovery under physiological conditions are not known. As reactive cysteine residues are typically crucial for catalytic activity, their oxidation leads to inactivation. Whether inactivation is just an unavoidable consequence of their reaction mechanism or serves a purpose for redox regulation remains to be explored.

Reactive cysteine residues in the catalytic site of mitochondrial enzymes as critical mediators of H_2O_2-dependent regulation

A large number of (abundant) mitochondrial enzymes, such as the thiolases and dehydrogenases described above, contain highly reactive cysteine residues without being 'professional' H_2O_2 scavengers (see above). In these proteins the cysteines are often part of the active site and may be able to serve as acceptor sites even at low H_2O_2 concentrations (Figure 6). In contrast to peroxidases the oxidized cysteines in these proteins may be more stable as they are often buried in substrate-binding groves whereas peroxidases have evolved to interact efficiently with their specific electron acceptors. It is unknown which fraction of H_2O_2 reacts with these enzymes but there is good evidence that transient oxidation of these enzymes can regulate their activity. As the reactive cysteine residues typically contribute to catalytic activity their oxidation leads to inactivation. These enzymes can in turn sense ambient H_2O_2 levels directly and translate this information into a change in activity. It will be an exciting task to study the physiological relevance of such a thiol switch-mediated redox sensing in mitochondria in the future.

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