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FORUM REVIEW ARTICLE

Mitochondrial Thiols in Antioxidant Protection and Redox Signaling: Distinct Roles for Glutathionylation and Other Thiol Modifications

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Abstract

Significance: The mitochondrial matrix contains much of the machinery at the heart of metabolism. This compartment is also exposed to a high and continual flux of superoxide, hydrogen peroxide, and related reactive species. To protect mitochondria from these sources of oxidative damage, there is an integrated set of thiol systems within the matrix comprising the thioredoxin/peroxiredoxin/methionine sulfoxide reductase pathways and the glutathione/glutathione peroxidase/glutathione-S-transferase/glutaredoxin pathways that in conjunction with protein thiols prevent much of this oxidative damage. In addition, the changes in the redox state of many components of these mitochondrial thiol systems may transduce and relay redox signals within and through the mitochondrial matrix to modulate the activity of biochemical processes. ***Recent Advances:*** Here, mitochondrial thiol systems are reviewed, and areas of uncertainty are pointed out, focusing on recent developments in our understanding of their roles. ***Critical Issues:*** The areas of particular focus are on the multiple, overlapping roles of mitochondrial thiols and on understanding how these thiols contribute to both antioxidant defenses and redox signaling. ***Future Directions:*** Recent technical progress in the identification and quantification of thiol modifications by redox proteomics means that many of the questions raised about the multiple roles of mitochondrial thiols can now be addressed. *Antioxid. Redox Signal*. 16, 476–495.

Introduction

The mitochondrial matrix is a distinct compartment with different properties from the rest of the cell. The presence of the components of the oxidative phosphorylation and other core metabolic machineries generates a flux of superoxide that is largely converted to hydrogen peroxide by the action of manganese superoxide dismutase (MnSOD) (6, 21, 55, 145). This hydrogen peroxide in the presence of ferrous or cuprous ions can generate the very reactive hydroxyl radical. Nitric oxide ($NO^{\bullet}$) can also diffuse into mitochondria and react with superoxide to generate the reactive and damaging species peroxynitrite (127, 142). Through these mechanisms, mitochondria are a significant source of a number of potentially damaging reactive oxygen species (ROS) (6, 21, 55, 145). Mitochondria also contain components that are susceptible to oxidative damage, including protein, DNA, and lipid. The extensive mitochondrial inner membrane is particularly prone to oxidative damage, because it contains a high proportion of unsaturated fatty acids (42). The ensuing lipid peroxidation can render the membrane more permeable to protons, thereby uncoupling oxidative phosphorylation, and can also disrupt the activity of the many enzymes and transporters embedded in the membrane. Lipid peroxidation also generates reactive aldehyde derivatives such as 4-hydroxynonenal that can damage mitochondrial proteins and DNA, both of which are also susceptible to direct attack by the hydroxyl radical and peroxynitrite. Further, some iron–sulfur centers in mitochondrial proteins such as aconitase are susceptible to damage by a direct reaction with superoxide. The accumulation of oxidative damage to mitochondria by these and other mechanisms is a significant component of many human pathologies, as it disrupts cell function by preventing ATP supply to the cell and increases the chances of cell death by apoptosis or necrosis

In addition to their role as damaging agents in pathology, some ROS, notably hydrogen peroxide, can act as redox signals both within mitochondria and between the organelle and other parts of the cell (6, 38, 49, 57, 93, 145, 223). These redox signals to and from mitochondria may be important modes of regulation and integration of metabolism, particularly as the mitochondrial matrix contains many of the central processes of metabolism, including oxidative phosphorylation, the citric acid cycle, fatty acid oxidation, the urea cycle, and the

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biosynthesis of iron sulfur centers and heme. In addition, the uptake of calcium into the matrix is an important aspect of cellular calcium homeostasis (50).

A range of mitochondrial thiol systems is present in the mitochondrial matrix and these are central to protecting mitochondria against oxidative damage and are thought to be a major mode by which potential redox signals are transmitted, modulated and sensed (41, 86, 87, 181). Here, what is known about mitochondrial thiol systems is surveyed, and their potential roles in protection and redox signaling are discussed. The focus of this article is the mitochondrial matrix in mammalian systems, and the many interesting aspects of thiol homeostasis in the mitochondrial intermembrane space (79) will not be considered here. The emphasis throughout is to indicate what is reasonably well established, point to possible unifying hypotheses and novel modes of action, and develop testable ideas that will enable us to learn more about how these systems operate *in vivo*.

Thiol Systems in the Mitochondrial Matrix

An important consideration of the many components of the mitochondrial matrix thiol system is that these thiols can act both in series and in parallel to modulate and respond to mitochondrial oxidative stress and redox signals (69, 97, 98). First, what is known about the individual components of the mitochondrial thiol systems (Figs. 1–3) is outlined and then how they may act in concert is shown (Fig. 4).

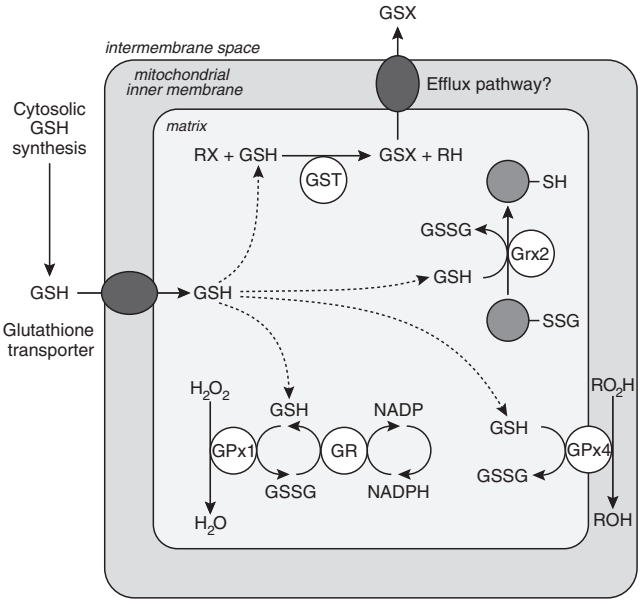

FIG. 1. The mitochondrial glutathione (GSH) system. This figure shows the import of GSH into mitochondria, its oxidation to glutathione disulfide (GSSG) by a range of processes including the action of glutathione peroxidase 1 (Gpx1) on hydrogen peroxide and the action of Gpx4 on phospholipid hydroperoxides (RO_2H), the reduction of GSSG by glutathione reductase (GR) and the reaction of GSH with electrophiles (RX) catalyzed by glutathione-S-transferases (GST), and the exchange of GSH with protein thiols catalyzed by Grx2.

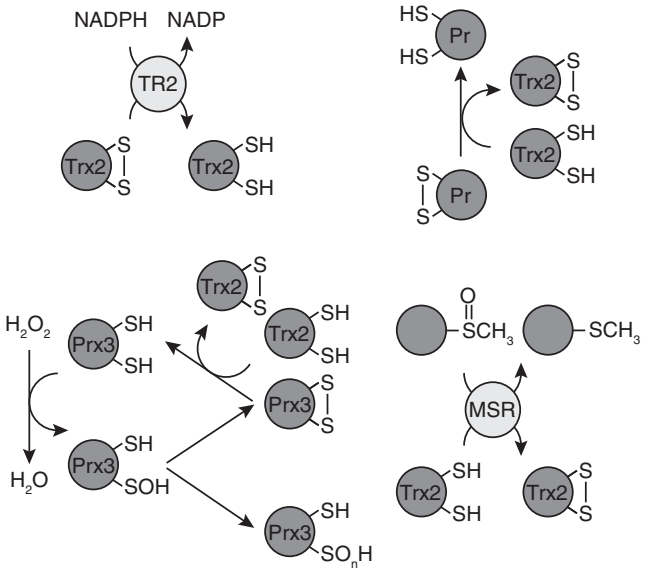

FIG. 2. The mitochondrial thioredoxin (Trx) system. Within mitochondria, Trx2 is reduced by the action of thioredoxin reductase 2 (TR2). Trx2 can then reduce disulfides on generic proteins (Pr). Among its other functions, it acts as the reductant for peroxiredoxins (Prxs) such as Prx3 and for methionine sulfoxide reductases (Msr) within mitochondria.

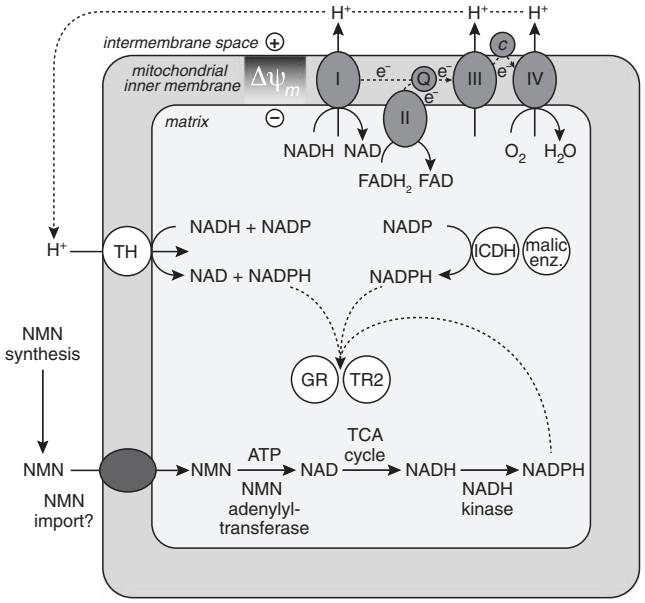

FIG. 3. The mitochondrial NADPH pool. A plausible, but unproved, scenario for how the mitochondrial NADPH pool is generated in mammals is shown. Nicotinamide mononucleotide (NMN) is synthesized in the cytosol and then imported by a putative transporter. In the matrix, NMN is converted to NAD by an adenylyl transferase reduced to NADH by the action of the tricarboxylic acid (TCA) cycle, and then converted to NADPH by a kinase. NADP is reduced to NADPH by the action of isocitrate dehydrogenase (ICDH) and malic enzyme. In addition, the transhydrogenase (TH) uses the membrane potential generated by the respiratory chain to drive NADP reduction by NADH.

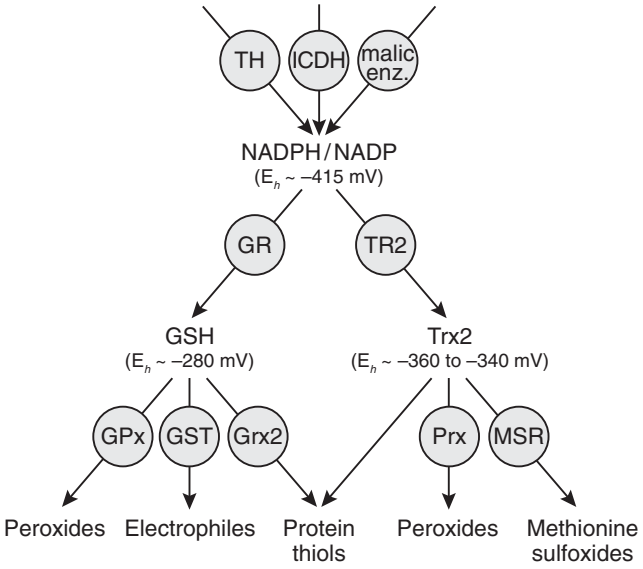

FIG. 4. Integrating the mitochondrial protein thiol system. This figure shows how both the mitochondrial GSH and Trx2 systems in mitochondria are reduced by the NADPH pool *via* glutathione reductase (GR) or TR2, respectively. The NAPDH pool is itself maintained by the action of the TH, ICDH and malic enzyme. The GSH then acts through Gpx, GST, and glutaredoxin (Grx2). The Trx2 pool acts through Prxs and Msr.

The mitochondrial glutathione system

Mitochondrial glutathione (GSH) is synthesized in the cytosol and then relatively slowly transported into mitochondria (Fig. 1) (54, 73, 101, 134, 167, 181). Uptake may be mediated by two members of the mitochondrial carrier family, the dicarboxylate carrier, and the 2-oxoglutarate carrier (27, 54, 112, 136). However, much remains uncertain about the nature and regulation of mitochondrial GSH transport, and the means by which mitochondria maintain their GSH concentration of $\sim$1–5 m*M* are unclear. Inside mitochondria, the GSH pool is primarily (95%–99%) in the reduced, GSH form with a small amount of glutathione disulfide (GSSG) (73, 181) (Fig. 1). The reduction potential of the mitochondrial GSH pool is more negative than that in the cytosol, with an E_h of -280 mV in the mitochondrion, compared with -260 to -200 mV in the cytosol (69). Although E_h in isolated mitochondria can be as low as -330 mV, it is unclear whether that is physiologically relevant (69). The mitochondrial GSH pool is maintained and reduced by the action of the flavoenzyme glutathione reductase (GR) (103, 194), which reduces mitochondrial GSSG to GSH using NADPH. There does not appear to be a mitochondrial GSSG efflux pathway (155), and the redox states of the mitochondrial and cytosolic GSH pools vary independently (137).

One important role of GSH is to degrade peroxides by the action of glutathione peroxidase (Gpx) enzymes with the concomitant conversion of GSH to GSSG. There are two forms of Gpx in the matrix: soluble glutathione peroxidase1 (Gpx1) (53) mainly degrades hydrogen peroxide. Glutathione peroxidase 4 (Gpx4) is adsorbed to the matrix surface of the inner membrane where it degrades phospholipid hydroperoxides to alcohols, and the resulting hydroxylated fatty acids are then cleaved from the phospholipid by phospholipases (2, 70, 90). This slows the propagation of lipid peroxidation within the mitochondrial inner membrane.

Glutathione-S-transferases (GSTs) use the nucleophilic thiol of GSH to detoxify electrophiles including xenobiotics and the products of endogenous oxidative damage such as α,β unsaturated aldehydes, quinones, epoxides, and alkyl hydroperoxides (62, 77). The dominant GST found in the mitochondrial matrix is GST-K, which is present as a soluble homodimer (77, 99, 160). There are also reports of GST-π (71) and GSTz1 within mitochondria (119), but their significance is not clear. GST-K is highly expressed in tissues such as the liver and kidney that are involved in detoxifying xenobiotics (77, 99, 160). However, it is also expressed in many other tissues such as the heart and brain that are not exposed to xenobiotics to the same extent, thus suggesting that this GST also detoxifies the products of mitochondrial oxidative damage (77, 99, 160). The products of these GST-catalyzed reactions are thioethers comprising the electrophile linked to GSH, and these are presumably excreted from mitochondria, although how this occurs is not known.

Glutaredoxins (Grx) catalyze the deglutathionylation of protein-GSH mixed disulfides far more effectively than other thiol proteins such as thioredoxin (Trx) (100). The Grx present in the mammalian cytosol, Grx1, is similar to the well-studied *Escherichia coli* enzyme, and both have a CPYC motif as the active site, which in *E. coli* contains a solvent-exposed Cys 11 and a buried Cys 14 (18). Cys 11 is sufficient for the deglutathionylation of a protein-GSH mixed disulfide, facilitated by the adjacent GSH binding site and its low pK_a (18, 72). This reaction transfers the GSH from the protein to Cys 11, which then reacts with another GSH to form GSSG, leaving a free Cys 11. Occasionally, the GSH on Cys 11 is displaced by the Cys 14 thiol leaving an intramolecular disulfide that can also be reduced by GSH. Mitochondria have their own Grx, glutaredoxin 2 (Grx2) (68, 72, 124), which is present at about 1 μM in the matrix (61). The Grx2 GSH binding site and the mechanism of mitochondrial Grx2 are similar to the cytosolic and *E. coli* enzymes; however, Grx2 has a CSYC motif at its active site with Cys 70 playing the key role in deglutathionylation (68, 96, 124). Grx1 has an additional exposed Cys residue that is readily modified by oxidants; however, Grx2 lacks this Cys residue, perhaps rendering it less easily inactivated by oxidative stress, S-nitrosating agents, and GSSG within the mitochondrial matrix (68, 76, 124). Further, Grx2 can be reduced directly by thioredoxin reductase 2 (TR2) (61, 96); however, this is far less efficient than reduction by GSH, which is more effective even at GSH levels 10% of normal (61); consequently, this mechanism is unlikely to play a physiologically important role in Grx2 reduction.

Together, these factors may enable Grx2 to operate effectively in a more oxidatively stressed environment than is the case for Grx1. The role of Grx2 is to catalyze the formation and reversal of protein-GSH mixed disulfides, which are important reactions of both protein thiols, and GSSG in antioxidant defense and in redox signaling (8, 58–60, 197). A further possibility is that this interplay between protein thiols and GSSG catalyzed by Grx2 enables protein thiols to buffer the GSH pool by converting GSSG to GSH while forming a protein-GSH mixed disulfide (40, 181, 183, 197). This might

enable the GSH/GSSG ratio to be maintained during transient periods of oxidative stress with the gradual reduction of the protein-GSH mixed disulfides after the oxidative stress has passed. Grx2 can form an inactive dimer around an iron sulfur center, and this dimer formation can be reversed by oxidative stress, thus potentially enabling the activation of Grx2 in response to elevated mitochondrial oxidative stress (95, 121, 141). Grx2 also has dehydroascorbate reductase activity (124), although with lower specific activity than Grx1, and may, thus, help recycle mitochondrial ascorbate. In addition, Grx2 is able to recycle the mitochondrial peroxidase, peroxiredoxin 3 (Prx3), after it has degraded a peroxide (74). Although Grx2 is the major Grx in mitochondria, Grx5 is also present, but it seems to be mainly involved in FeS center biosynthesis (205, 215).

There are other enzymes that utilize GSH to detoxify reactive species. One is the glyoxalase system that detoxifies dicarbonyl products of carbohydrate metabolism, particularly methylglyoxal (143, 164). These compounds cause the nonspecific glycation and damage to a range of mitochondrial components (143, 164). In this pathway, methyl glyoxal reacts with GSH to form an unstable hemithioacetal that is isomerized to S-D-lactoyl GSH by the action of glyoxalase I and is then hydrolyzed by glyoxalase II to release D-lactate and GSH (164). Although there is evidence that methylglyoxal damages mitochondria and that glyoxalase I protects against this damage (16), glyoxalase I seems to be found only in the cytosol (111). Although glyoxalase II is found in the mitochondria (31), perhaps this occurs because it has other functions there, such as the hydrolysis of GSH thioesters, distinct from its role in glyoxal detoxification. The properties of the GSH system are summarized in Figure 1. Within mitochondria, there are other low-molecular-weight thiols such as cysteine and free lipoic acid that contribute to the overall thiol content of the matrix. However, these thiols are present in far lower concentrations than that of GSH and the general assumption is that they are of relatively marginal importance for antioxidant defense (75).

The mitochondrial Trx system

Trx is a ubiquitous small ($\sim$12 kDa) protein that has a dithiol WCGPC motif in its active site that transduces redox processes in a range of biochemical reactions (84, 122). Within the mitochondrial matrix, there is a distinct Trx system centered on Trx2 (4, 84, 122, 187, 195) (Fig. 2). The reaction of Trx2 is typically reduction of disulfide bonds on other proteins, and for this, an active thiol in Trx2 acts as a nucleophile to attack the target disulfide bond forming a transient disulfide bridge with the substrate protein. This intermediate is then reduced by the attack of the second, or resolving, thiol of Trx2, thereby reducing the target protein's disulfide. The disulfide left on Trx2 is then recycled back to the dithiol by the mitochondrial selenoenzyme TR2 that utilizes NADPH (63, 113, 139). TR2 can also reduce dehydroascorbate to ascorbate and may, thus, play a role in recycling the mitochondrial ascorbate pool (120). The E_h of the mitochondrial Trx2 pool is -360 to -340 mV, which is significantly more reducing than that in the cytosol (69). Trx can also reduce protein-GSH mixed disulfides but does so less effectively than Grx (157).

A vital role for Trxs is as the reductant of Prxs, which are ubiquitous peroxidases that degrade hydrogen peroxide and alkyl peroxides (172, 173). Typically two cysteine Prxs are dimeric proteins containing an active thiol that reacts rapidly with hydrogen peroxide to generate a sulfenic acid (172, 173). This sulfenic acid then reacts with an adjacent thiol on the other member of the dimer to form an interprotein disulfide (172, 173). This disulfide is then reduced to the dithiol form by a reaction with Trx (172, 173). In the mitochondrial matrix, the predominant Prx is Prx3 (203) with lower amounts of Prx5 also present, and Prx5 is also found in the cytosol (172, 173). Interestingly, Grx2 is also able to recycle Prx3, but not Prx5, with similar kinetics as Trx2 (74). Since Prx3 is present in the matrix at high concentrations ($\sim 60\,\mu M$) (36) and rapidly reacts with hydrogen peroxide ($\sim 2\times10^7\ M^{-1}s^{-1}$) (34), it is a major sink for hydrogen peroxide within mitochondria, degrading as much as 90% of the hydrogen peroxide produced in the matrix (36). Prx5 is an atypical Prx, as it forms an intraprotein disulfide as a part of its reaction cycle, rather than an interprotein disulfide. Prx5 also degrades hydrogen peroxide but does so more slowly than Prx3 ($3\times10^5\ M^{-1}s^{-1}$) (36); however, Prx5 degrades peroxynitrite rapidly ($7\times10^7\ M^{-1}s^{-1}$) (199), and this has been suggested as an important role for Prx5 *in vivo* (36).

A significant constraint on these estimates of Prx3 peroxide consumption is that Prx3 activity is affected by exposure to hydrogen peroxide and other ROS. This happens in two ways: hydrogen peroxide converts Prx3 to an intersubunit disulfide that has to be converted back to the active dithiol form by Trx2; consequently, a significant proportion of Prx3 may be in the inactive dimeric state. A second mechanism by which Prx3 activity is affected by hydrogen peroxide occurs, because the sulfenic acid on the active Prx cysteine is vulnerable to hyperoxidation to a sulfinic acid and then to a sulfonic acid, neither of which can be reduced back to the active form by Trx2. Interestingly, the C-terminal of mammalian Prxs slows disulfide formation making hyperoxidation more likely, perhaps to facilitate the regulation of hydrogen peroxide signaling (211). The sulfinic acid, thus, formed on many Prxs, but not the sulfonic acid, can be reduced back to the thiol by sulfiredoxin (Srx) (210). Although Srx is mainly active in the cytosol, under oxidative stress, it can translocate to mitochondria and there regenerate Prx3 (152); even so, mitochondrial Prxs are more susceptible to hyperoxidation than those in the cytosol (5). The activity of Prx3 may also be affected by its oligomeric state, as it can form dodecameric toroids that stack into filaments under reducing conditions (7, 36). Further, some hyperoxidized Prxs can form toroids with an apparent chaperone function, but it is not known whether this happens for Prx3 (36). Finally, there is the possibility that Prx activity can be modified by phosphorylation (3), but the significance of this within mitochondria is unclear.

The sulfur on methionine residues is susceptible to oxidation to a methionine sulfoxide, and the mitochondrial Trx system helps reverse this damage through methionine sulfoxide reductases (MSRs) (105). Since the sulfur atom in methionine sulfoxide is chiral, there are separate MSRs for the *R* and *S* enantiomers, with MsrA reducing the *S*-form and MsrB reducing the *R*-form (105). In mammals, there is a single MsrA isoform that is found in both the cytosol and

the mitochondrial matrix (201); whereas there are three MsrB isoforms, one of which, MsrB2, is localized to mitochondria (104). The mitochondrial forms of both MsrA and MsrB2 act through a catalytic cysteine residue that reduces the methionine sulfoxide to a methionine and generates a sulfenic acid on the Msr that subsequently forms an intraprotein disulfide which is then recycled by Trx2. Thus, MsrA and MsrB2, in conjunction with Trx2, have important roles in preventing oxidative damage to mitochondrial proteins (149, 216).

Mitochondrial protein thiols

There are cysteine residues on the surface of proteins that contain exposed thiols which are free to interact with the aqueous solvent (131, 133, 181). These thiols are distinct from those with well-established functions, such as in enzyme active sites, or with structural roles in iron sulfur centers, as thioether links to hemes or in Zn finger motifs (177, 181). One potential structural role for thiols on protein surfaces is to form intra- and interprotein disulfide bridges; excreted proteins, extracellular protein domains, and proteins that reside in the endoplasmic reticulum frequently incorporate disulfides to enhance stability, whereas disulfide bridges are rare in intracellular proteins (140, 177). Thus, solvent-exposed thiols on protein surfaces have no evident function and, in fact, tend to be selected against (131). The concentration of exposed thiols on the surface of native proteins within isolated mammalian mitochondria is ~60–90 m*M* (169), ~20–25-fold higher than the concentration of GSH, which is the next most abundant free thiol (169). It is an underappreciated fact that within the mitochondrial matrix, and most probably elsewhere in the cell, exposed thiols on protein surfaces are the quantitatively dominant thiol (75, 100, 169, 197). However, since each protein thiol is a distinct species, the collection of small concentrations of individual protein thiols may be less effective at driving reactions than the homogeneous GSH pool, which has a mass action advantage.

The abundance of thiols on protein surfaces is intriguing, because sulfur is a relatively low abundance element making it metabolically costly to use cysteine instead of another otherwise similar amino acid, such as a serine, on protein surfaces (37). In addition, exposed surface cysteines are reactive and tend to be eliminated by evolution if they do not have a function (131). This is supported by the fact that the abundance of cysteine residues in proteins is less than would be expected by chance when codon usage is corrected for, thus suggesting that those cysteines which are retained have a functional role (140). Further, the proportion of cysteine residues in protein increases with organismal complexity (140) with 92% of proteins in mammals having at least one cysteine, whereas only 50% of proteins in *Archea* have a cysteine. Together, these data suggest that the abundant thiols exposed on the surface of protein may be there for a reason (131). The potential function(s) of surface protein thiols will depend on their properties, which, in turn, are dictated by their local environment. For most protein thiols, the pK_a is in the range 8–9 (204), although exposed single-protein surface thiols seem to have a lower pK_a and will, thus, be more reactive (131). This is important, because most potential antioxidant or regulatory thiol reactions either occur through the thiolate anion, or are faster for the thiolate. Since the pH of the mitochondrial matrix is higher than that of the cytosol (~7.8–8 against 7.2), free thiols will be up to sixfold more reactive in mitochondria than in the cytosol. Thiol reactivity can also be altered by changing the pK_a in response to the local environment of the cysteine residue, and values down to 3.5 have been reported for protein thiols with dramatic consequences for their reactivity (46, 65, 66, 92, 204). Although no studies have focused systematically on the range of pK_a values of surface mitochondrial thiols or the range of their reactivities, a reasonable working assumption is that the majority are of broadly similar reactivity with pK_a values in the "normal" range of 8–9 (although these surface thiols may have a lower pK_a on average) (131), with a small proportion significantly altered in reactivity, pK_a and stability of post-translational modifications due to local factors such as the proximity of basic or acidic amino acids (8, 46, 133, 177, 204). This assumption is consistent with studies in which only a few percent of surface protein thiols are more susceptible to persistent modifications such as S-nitrosation or glutathionylation (8, 29, 88, 163), and with global assessments of cysteine reactivity in proteomes (204). In addition to the pKa, other factors affect the reactivity of proteins thiols, including how accessible the thiol is to potential reaction partners (40). The reactivity of protein thiols is also constrained by the orientation of the thiol relative to its reaction partner, as S_N2 nucleophilic attack by a thiolate on an electrophile is favored by a transition state in which the attacking and leaving groups, and the electrophilic center, are in a straight line (64–66). Similar constraints affect the stability of many modifications to surface protein thiols such as glutathionylation, disulfide formation, or S-nitrosation, which depend on the ability of an attacking nucleophile to displace the modification from the cysteine residue (64–66). Thus, the reactivity and the on/off rates for the posttranslational modification of particular thiols will vary significantly depending on their local environment.

Surface protein thiols so far have been considered in isolation. However, a significant proportion of surface protein thiols occur close enough to another thiol to be able to form an intramolecular disulfide, and these are called vicinal dithiols (67, 83). Vicinal dithiol pairs can occur due to a -CX_nC- motif in the primary sequence, where n is typically 2–6 (67). Genome analysis suggests that the proportion of (-CX_2C-) motifs is higher than expected by chance with 20% of human proteins containing such a structure (140), although it is not clear to what extent this reflects the use of vicinal dithiols in structural motifs such as iron sulfur centers. Further, there is evidence that surface thiols tend to cluster together (131). Vicinal dithiols can also occur through proximity in the tertiary or quaternary structures and in some cases, the crystal structure has enabled the presence of a vicinal dithiol not found in the primary sequence to be confirmed (168). The existence of vicinal dithiols on proteins has been determined experimentally using arsenical reagents that react selectively with vicinal dithiols, and these have shown that about 5% of soluble proteins in lymphoblasts contain vicinal dithiols (67) and that in isolated mammalian mitochondria, about 5%–15% of total exposed protein thiols are a part of a vicinal dithiol pair (168).

Vicinal dithiols have properties that are useful and distinct from single-protein thiols, and this may be why clustering of surface protein thiols is favored (131). The

very high local thiol concentration makes vicinal dithiols more reactive with each other, and, thus, more reducing, than is the case for lone surface thiols (65, 181). This greater tendency of surface vicinal dithiols to form a disulfide may be useful by facilitating the recycling of these thiols when they participate in antioxidant reactions and may also act as redox switches to transduce the response of the protein to redox signaling by altering protein structure and/or function (67, 181).

To summarize, there is a high concentration of thiols on protein surfaces and within the mitochondrial matrix, these are the quantitatively dominant free thiol. In addition, a significant proportion of these thiols are vicinal dithiols which have properties that may favor their use in antioxidant defense and redox signaling. The single thiols are more likely to represent sites for S-nitrosation, glutathionyation, and sulfenic acid formation, and these are likely to be recycled by Grx2; whereas when the vicinal dithiols are converted to a disulfide, these are likely to be recycled by the Trx system. Most of these thiols are likely to have similar reactivities, with a small proportion being particular reactive. Protein thiols may play important but underappreciated roles in antioxidant defense and redox signaling.

The mitochondrial matrix NADPH pool

The activities of the mitochondrial GSH and Trx pools require them to be in their active, reduced forms. Consequently, GSSG and oxidized Trx2 are rapidly reduced by NADPH through GR or TR2, respectively, thus making the mitochondrial NADPH pool central to defense and signaling by the mitochondrial thiol systems (11). To drive these reactions, the mitochondrial NADPH/NADP pool is kept reduced with an E_h of about -415 mV (69, 185). This contrasts with the mitochondrial NADH/NAD pool which has a variable redox state that is always oxidized relative to the NADPH/NADP pool, with a typical E_h of around -300 mV (69, 185) (Fig. 3). However, the E_h values of these two pools have not been extensively studied in a wide range of tissues; so, these estimates may vary (69, 185).

In yeast and plants, NAD is synthesized in the cytosol and is then imported into mitochondria by a specific carrier (198). In the matrix, the NAD is then reduced to NADH and converted to NADPH by a specific kinase (156). In mammals, the mitochondrial NADPH/NADP pool also originates from the cytosol, but it seems likely that in this case, it is the nicotinamide adenine mononucleotide that is imported, as no homolog of the yeast NAD carrier is found in mammals (150). Further, there is a mitochondrial nicotinamide adenine mononucleotide adenylyltransferase (221) that will form NAD from the imported mononucleotide which is likely to then go on to form NADPH as in yeast (11).

Within mitochondria, there are two distinct pathways that drive the reduction of NADP to NADPH. The first is by the action of NADPH dependent dehydrogenases, including isocitrate dehydrogenase (94, 178) and malic enzyme (162), with a possible role for aldehyde deydrogenases (162). The second pathway of NADP reduction is through the mitochondrial transhydrogenase, which is a membrane protein embedded in the mitochondrial inner membrane that uses the proton motive force across the inner membrane to drive the reduction of NADP to NADPH using electrons from NADH (12, 178). Together, these processes maintain the mitochondrial NADPH pool in a highly reduced state (Fig. 3) (69, 185).

Integration Without Equilibration of Mitochondrial Matrix Thiol Systems

Mitochondrial thiols can be considered as two parallel systems that are both driven by the NADPH pool, *via* GR for the GSH system and *via* TR2 for the Trx system (Fig. 4). These two pathways then act through the Trx2 or GSH pools to drive the thiol redox processes that contribute to both antioxidant defenses and redox signaling. There is consequently considerable overlap and redundancy in dealing with oxidative processes and regulation. An important consideration is that the systems are not at equilibrium but are both spatially and kinetically compartmentalized (69, 97, 98). The redox environment within the mitochondrial matrix is independent of that in the cytosol; thus, the E_h values of both the GSH and Trx2 pools can vary considerably from those in the cytosol. Within the mitochondrion, the GSH and the Trx2 pools are kinetically separate and are not at equilibrium, with the Trx2 pool being significantly more reduced than that of GSH, as is clear on considering their E_h values (Fig. 4). This difference in E_h has significant implications for how we should think about these systems, as it implies that oxidative stress is not a global imbalance in ROS production and consumption, but that instead the disruption to each individual redox couple has to be determined and considered when assessing oxidative changes (98).

Although the mitochondrial GSH and Trx2 pools are kinetically compartmentalized, there are links between them. One is at the level of Prx3 that can be reduced by both Trx2 and Grx2. Finally, since both Trx2 and GSH/Grx2 can react directly with protein disulfides, the two pathways may influence and interact with each other *via* changes in exposed protein thiols.

Antioxidant Defense by Mitochondrial Thiols

Most of the thiol systems just outlined contribute to antioxidant defenses within the mitochondrial matrix. Although many of the major modes of antioxidant defense are reasonably well understood, other possible mechanisms have only been suggested based on theoretical grounds or on simple *in vitro* experiments; consequently, their significance *in vivo* is uncertain. Here, the ways that thiols can protect the mitochondrial matrix and distinguish those which are well established from those whose status is currently uncertain are surveyed.

Degradation of peroxides by antioxidant enzymes

The proximal ROS formed within mitochondria is superoxide, which is mainly converted to hydrogen peroxide by the action of MnSOD (145). Since hydrogen peroxide can cause oxidative damage in the presence of metals through the formation of the hydroxyl radical, or react directly with thiols to form sulfenic acids, its levels are tightly controlled through degradation by mitochondrial Prxs and Gpxs (36). The highest capacity mitochondrial Prx is Prx3, which, due to its high concentration and rapid reaction with hydrogen peroxide, contributes $\sim$90% of the hydrogen peroxide degrading

capacity of the matrix (36). However, this is the maximum capacity of Prx3, and a potential limitation is that Prx3 can be partially inactivated on exposure to excessive levels of hydrogen peroxide (172–174) (Fig. 2). This has been shown to occur in the heart during ischemia-reperfusion injury (110). Thus, under conditions of high hydrogen peroxide flux, such as may occur within mitochondria under pathological conditions, a significant fraction of Prx3 may be inactive (34, 35, 110). Prx5 reacts with hydrogen peroxide ~100-fold more slowly than Prx3 and is also present at a 3-fold lower concentration, thus making its role in degrading hydrogen peroxide comparatively minor (36). However, Prx5 does rapidly react with peroxynitrite ($7\times10^7\ M^{-1}s^{-1}$) (199), and since its matrix concentration is ~20 μM, this may be a significant role *in vivo* (36). The other main peroxidases in mitochondria are Gpx1, which selectively degrades hydrogen peroxide, and Gpx4 which preferentially degrades phospholipid peroxides (2). Gpx1 seems to be the only other significant potential enzyme for hydrogen peroxide degradation within mitochondria contributing ~9% of the potential capacity based on its concentration within the mitochondrial matrix (~2 μM) and its rate with hydrogen peroxide ($6\times10^7\ M^{-1}s^{-1}$) (36). Thus, Prx3 is the major sink for hydrogen peroxide within mitochondria, with Gpx1 being the only other significant contributor. However, the susceptibility of Prx3 to inactivation may make it less effective in many pathological conditions. Gpx1 may then become the major sink for mitochondrial hydrogen peroxide, although it too can be inactivated by hydrogen peroxide through irreversible alteration to its selenocysteine (28); however, whether this occurs in mitochondria is not known.

To assess the relative importance of Prxs and Gpxs is difficult, as they have overlapping targets; however, an indication can be obtained by comparing the relative effects of deleting these enzymes on susceptibility to oxidative damage *in vivo* (30). Mice lacking Prx3 have no overt phenotype but are more sensitive to lipopolysaccharide-induced damage (118). In mice overexpressing Prx3, there was an improvement in a number of pathologies including left ventricular remodeling after cardiac ischemia-reperfusion injury (135) and improved glucose tolerance in mice (25). Although there are no reported Prx5 mouse models, silencing Prx5 in cells in culture makes them more susceptible to oxidative damage (44). Mice lacking Gpx1 are viable and show little phenotypic change compared with wild type (81), but they are more susceptible to oxidative damage caused by paraquat or hydrogen peroxide (43). However, since Gpx1 is present in both the cytosol and the mitochondria, the importance of mitochondrial damage to this outcome is uncertain. In a different Gpx1 knock out mouse model, it was shown that mitochondria from the liver, but not the heart, had increased hydrogen peroxide production and oxidative damage (53). Homozygous knock out of Gpx4 is embryonic lethal, and heterozygotes are more susceptible to oxidative damage (214). This is consistent with the important role for Gpx4 in preventing lipid peroxidation to mitochondria in cell models (153, 154). Unsurprisingly, the ubiquitous silencing of TR2 or of Trx2 is embryonic lethal (30, 195), although heterozygous Trx2 (+/−) mice do not have any major phenotypic changes (195). Thus, we can conclude that preventing mitochondrial oxidative damage due to peroxides *in vivo* is mainly shared between Prx3 and Gpx4 with Gpx1 and Prx5 also contributing.

Protection against oxidative damage by other enzymes

The repair of methionine sulfoxide formation on mitochondrial proteins is carried out by MsrA and MsrB2. When MsrA is silenced in mice, they are more susceptible to mitochondrial oxidative damage (149). Conversely, overexpressing MsrA or MsrB2 in cells in culture is protective against oxidative damage (20, 158, 216). Mitochondrial oxidative damage leads to lipid peroxidation that generates reactive aldehydes such as 4-hydroxylnonenal, which, in turn, leads on to further damage to mitochondrial components (77). Mitochondrial GSTs can, in principle, protect against many of these electrophiles by conjugating them to GSH. This protective role is consistent with the finding that silencing the ortholog of GST-K in *Caenorhabditis elegans* disrupts mitochondrial function (161); however, the importance of GST-K in protecting mitochondria against oxidative damage is uncertain.

Direct antioxidant activity of protein thiols and GSH

The concentration of exposed thiols in the mitochondrial matrix on the surface of proteins is ~50 mM, and there is also a substantial concentration (~1–5 mM) of GSH (169). These thiols can react directly with a range of potentially damaging species, either by the thiol or thiolate donating electrons or hydrogen atoms to repair radicals, or by the thiolate reacting with electrophiles (202). Further, the higher pH of the mitochondrial matrix makes thiols more reactive than in the cytosol. Together, these points raise the possibility that free thiols may contribute to antioxidant defenses by direct, uncatalyzed reaction with damaging species (100, 169, 197).

To assess how the reactions of thiols may contribute to mitochondrial antioxidant defenses, the reactivity of these thiols with the various species that contribute to mitochondrial oxidative damage needs to be considered (208). The reaction rate of a thiol with superoxide is in the range 30–1000 $M^{-1}s^{-1}$ (209), far less that that of superoxide with MnSOD (~$2\times10^9\ M^{-1}s^{-1}$) (145); hence, the direct reaction of thiols will not contribute to superoxide degradation within mitochondria (208). The reaction of thiols, such as those of GSH and of "typical" protein thiols, with hydrogen peroxide is relatively slow (1–20 $M^{-1}s^{-1}$) (36). Thus, despite the high thiol concentration within mitochondria, the overall rate of decomposition is negligible compared with that by Prx3 (~$2\times10^7\ M^{-1}s^{-1}$) (36) or Gpx1 ($6\times10^7\ M^{-1}s^{-1}$), which are present at 60 and 2 μM, respectively (36). The reaction of thiols with peroxynitrite is faster than that with hydrogen peroxide [700 $M^{-1}s^{-1}$ with GSH (14) and 200–300 $M^{-1}s^{-1}$ with protein thiols (165)]. There are no confirmed pathways for peroxynitrite degradation within mitochondria, although Prx5 reacts with peroxynitrite at a rate of $7\times10^7\ M^{-1}s^{-1}$ (199) and is present at a concentration of 20 μM (36); hence, it has been suggested as a possible candidate. Comparing the maximal capacities for peroxynitrite degradation by protein thiols and Prx5 suggests that the role of exposed thiols is minor compared with Prx5 (36); however, whether Prx5 actually operates as a peroxynitrite sink *in vivo* is uncertain. The reaction of thiols with hypochlorous acid is rapid ($3\times10^7\ M^{-1}s^{-1}$) (159), thus suggesting that this may be a major factor in its degradation; however, the short half life and consequent limited diffusion of hypochlorous acid *in vivo* and the fact that it is

generated outside the cell suggests that it may rarely encounter mitochondria (208).

Both thiols and thiolates rapidly react with damaging free radicals, typically by transferring a hydrogen atom or an electron to generate a nonradical species and in doing so, the thiol is converted to a thiyl radical (180, 202). Although both thiols and thiolates can react with radicals, electron donation is faster from a thiolate (202); so, the increased reactivity that occurs for other thiol reactions within mitochondria will also occur for radical quenching. A range of potentially damaging free radicals can react with thiols in this way. Although the hydroxyl radical is rapidly quenched by thiols ($1–4\times10^{10}$ $M^{-1}s^{-1}$) (14, 106), its reaction with most biological molecules is similarly rapid; so, though the abundance of thiols make them a significant target for reaction with the hydroxyl radical, the diffusion limited reaction of the hydroxyl radical with other biomolecules means that thiols will not be able to act as effective antioxidants against this species. The nitrogen dioxide radical $NO_2^{\bullet}$, derived from $NO^{\bullet}$ metabolism, rapidly reacts with thiols ($3–5\times10^{7}$ $M^{-1}s^{-1}$) (56), and the rate of reaction of thiols with the carboxylate radical, $CO_3^{\bullet-}$, is also rapid (5×10^{6} $M^{-1}s^{-1}$) (14). Thus, a reaction with thiols may be a major sink for these radicals and related species in the mitochondrial matrix. Mitochondrial thiols can also react with electrophiles, such as the α,β unsaturated aldehydes derived from oxidative damage to lipids, although the rates are low (e.g., 1.2 $M^{-1}s^{-1}$ for 4-hydroxynonenal) (47). Even so, these reactive aldehydes alkylate proteins and DNA; so, their reaction with thiols may be protective. Since the products of these reactions are thioether derivatives of protein thiols, they may result in irreversibly damaged protein that has to be proteolytically degraded. In addition to these irreversible modifications, there are other damaging species such as methylglyoxal that can react reversibly with protein thiols to form a hemithioacetal (22). This may minimize the damage caused by these species until they can be eliminated. Through these reactions, exposed thiols within the mitochondrial matrix may complement the protective role of peroxidases by reacting with and degrading damaging radicals and electrophiles.

The reactions of exposed thiols with damaging species will generally convert the thiol to a thiyl radical or a sulfenic acid (40, 197). In the presence of oxygen, these species are unstable and tend to oxidize further to sulfinic and sulfonic acids (102, 189). Since these oxidations are generally irreversible, this would allow the thiol to participate in only one defensive reaction before being degraded and may also disrupt protein function. Most effective biological antioxidants undergo multiple defensive reactions, because they are rapidly recycled back to the active antioxidant after having quenched a damaging species. So, for free thiols to be effective antioxidants, the thiyl and sulfenic acid intermediates should be recycled back to the thiol (169, 197).

The reaction of a sulfenic acid on a protein with hydrogen peroxide is relatively slow (0.4 $M^{-1}s^{-1}$) (1, 200), but even so, the sulfinic acid product will prevent recycling of the cysteine residue. Oxidation of the sulfenic acid can be prevented by reaction with a thiolate to displace a hydroxide and form a disulfide. The reaction with GSH is relatively slow (2.9 $M^{-1}s^{-1}$) (1, 200); however, since the sulfenic acid also reacts slowly with hydrogen peroxide, this may be sufficient to recycle the thiol. Alternative ways in which sulfenic acid derivatives can be recycled is to react with an adjacent thiol in a vicinal dithiol, or with a protein nitrogen to form a sulfenyl amide (176, 186). This may explain why protein thiols on the surface of proteins tend to cluster (131).

The recycling of a protein thiyl radical has to be done far more rapidly and efficiently than for a sulfinic acid, because thiyl radicals react rapidly ($\sim2\times10^{9}$ $M^{-1}s^{-1}$) (202) with oxygen to form a thioperoxyl radical that can then be further oxidized. The thiyl radical can also abstract $H^{\bullet}$ from other amino acid residues in the protein relatively rapidly ($10^{3}–10^{5}$ $M^{-1}s^{-1}$) (180) and, thus, cause intraprotein oxidative damage. To stop the irreversible oxidation of the protein, it is important to recycle the protein thiyl radical before these reactions occur (180, 202). A major recycling pathway is by the reaction of the thiyl radical with a thiolate to form a radical disulfide, which occurs rapidly ($5\times10^{8}–5\times10^{9}$ $M^{-1}s^{-1}$) (202). The radical disulfide anion formed then rapidly loses its electron to oxygen ($\sim5\times10^{9}$ $M^{-1}s^{-1}$) (202) to form superoxide by the Winterbourn reaction (180, 207), thus enabling the superoxide to be dismutated by MnSOD and the hydrogen peroxide to be degraded by peroxidases. Since thiolates react with thiyl radicals faster than the thiol, this reaction will be accelerated at the elevated pH of the matrix compared with the cytosol (180, 202). The thiol that reacts with the thiyl radical can be provided by a GSH to form a mixed disulfide, or when the protein thiyl is a part of a vicinal dithiol pair, the reaction with the adjacent thiol will form an intraprotein disulfide.

Other reactions can be potentially used to recycle protein thiyl radicals. Thiyl radicals are rapidly recycled by ascorbate (6×10^{8} $M^{-1}s^{-1}$ at pH 7) (180, 202), and mitochondrial ascorbate is in the range 100–500 μM (120). Thiyl radicals can also be recycled by the rapid reaction with free $NO^{\bullet}$ to form an S-nitrosothiol ($2–3\times10^{9}$ $M^{-1}s^{-1}$) (128). Protein S -nitrosothiols can be recycled back to free thiols by a reaction with thiolate to displace the nitroxyl radical (NO^{-}) and leave a disulfide, or the nitrosonium (NO^{+}) may be transferred to other thiols regenerating the thiol (32, 82, 93, 190). This may contribute to the ability of $NO^{\bullet}$ to act as an antioxidant (206), although evidence for this *in vivo* is indirect at present. It is unclear whether there are mitochondrial proteins that can react with a protein thiyl radical, but some cytosolic protein thiyls can react with Prxs, and this may recycle them (217). Since the reaction of GSH with many protein sulfenic acids is slow (2.9 $M^{-1}s^{-1}$) (1, 200), it may be advantageous for this reaction to be enzyme catalyzed. GSTs catalyze the glutathionylation of sulfenic acids on proteins such as Prx6 (77, 130), but whether a similar reaction is catalyzed by mitochondrial GSTs is not known. Neutralization of radicals by the thiol of GSH will generate a sulfenic acid or a thiyl radical on GSH, which can be most easily recycled by reaction with a protein thiol to form a glutathionylated protein, or with another GSH to form GSSG, for recycling by GR. Alternatively, the $GS^{\bullet}$ can directly react with Grx to form a glutathionylated Grx (61, 191), and thereby recycle the thiyl radical to GSSG.

To complete regeneration of the protein thiol system, the disulfides formed should be recycled. For vicinal dithiols, the disulfide can be returned to the dithiol form by reaction with Trx2 (100, 157). GSH can also react with disulfides to reduce them to a dithiol (100, 157), and this reaction is greatly accelerated by Grx2. Glutathionylated protein thiols can be

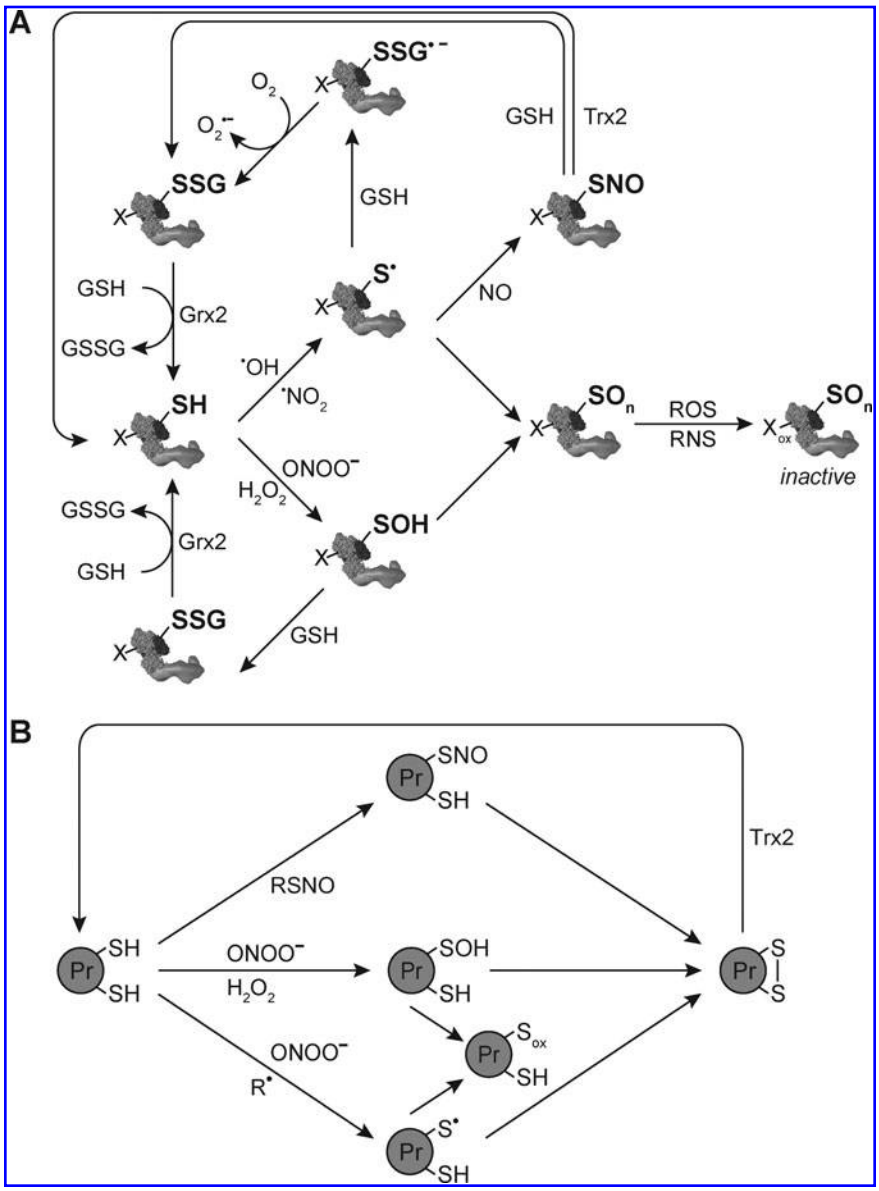

FIG. 5. Protection against oxidative damage by mitochondrial protein thiols. (A) A thiol on the surface of a protein being converted to a thiyl radical by reaction with damaging nitrogen dioxide or hydroxyl radicals, or to a sulfenic acid by reaction with hydrogen peroxide or peroxynitrite. These modifications are then recycled back to a thiol by GSH in conjunction with Grx2. The thiyl radical can also be recycled by a reaction with NO to form a nitrosothiol that is then recycled to the thiol by GSH or Trx2, or by a reaction with ascorbate. If the thiyl radical or sulfenic acid is not repaired, then it goes on to form a higher oxidation state. This prevents the thiol from protecting an adjacent amino acid (X), which, in turn, becomes oxidatively damaged. **(B)** The interactions of a vicinal dithiol pair with oxidants and radicals ($R^{\bullet}$) is shown. Here, it can be seen that the oxidized thiols are recycled by forming a vicinal disulfide which is then reduced back to the vicinal dithiol by the action of GSH/Grx2 or Trx2.

rapidly deglutathionylated with GSH catalyzed by Grx2 (61, 96).

The reactions just outlined may enable protein thiols, in conjunction with GSH, $NO^{\bullet}$, Grx2, and Trx2, to be a concerted antioxidant cycle protecting all components of the mitochondrial matrix from oxidative damage, as was originally proposed by Thomas (197) (Fig. 5). This is similar to the mechanism of protection proposed for MSRs (115, 126). The rapid recycling of oxidized protein thiols may act as a sink for reactive species that are not dealt with by enzymatic antioxidants. This mechanism will prevent protein thiols from becoming irreversibly oxidized, but may also prevent oxidative damage to other amino acid residues, in addition to cysteine. The simplest way in which this would occur is by locating cysteine residues close to vulnerable but essential amino acid residues so the cysteine can react preferentially with the damaging species (89). This is analogous to a role proposed for methionine residues that can be "sacrificially" oxidized to a methionine sulfoxide which is then reduced back by the action of Msr (115, 116, 125). Oxidative damage to critical amino acids may also be repaired by nearby cysteine residues. This can occur because oxidative damage often entails transient formation of a radical on an amino acid residue. These radicals could be repaired by electron donation from a nearby cysteine residue, before the radical reacted further to form an irreversibly oxidized product. This possibility is supported by work from the Kalyanaraman lab which showed that intramolecular electron transfer between cysteine residues and a tyrosyl radical rapidly occurs (10^3–10^4 $M^{-1}s^{-1}$) within model peptides (220). Since electrons can tunnel up to 14 Å within proteins (144), this mechanism may allow surface cysteine residues to repair a range of protein radicals and thereby decrease protein oxidative damage. The thiyl radical, thus, formed would then be recycled back to the thiol form by the mechanisms just outlined.

In summary, a plausible case can be made for surface thiols on mitochondrial proteins contributing to antioxidant defenses. Under oxidative stress, there is an accumulation of

glutathionylated proteins, vicinal disulfides, and S-nitrosothiols, which is consistent with this model (58–60, 86, 155, 166, 168, 179). Further, blocking thiols on mitochondrial membranes makes the membranes more susceptible to oxidative damage (169). There is also a large body of evidence that Grx2 is an important antioxidant within mitochondria, as decreasing its expression increased oxidative damage (123) while overexpressing Grx2 protects mitochondria (52, 135, 148, 212). Further, when isolated mitochondria are exposed to hydrogen peroxide, there is glutathionylation of mitochondrial proteins even when there is negligible oxidation of the GSH pool (89). Together, these findings are consistent with Grx2 and GSH playing a protective role by recycling oxidized protein thiols within mitochondria.

However, it should be noted that the components of these systems have multiple effects on a range of antioxidant defense systems; consequently, there are other possible modes of antioxidant defense that could account for these findings. The very large number of surface thiols in mitochondria makes altering the bulk amount impractical, particularly as agents that block protein surface thiols disrupt other enzymes and transporters. It may be possible to use bioinformatic analyses to test the implications of the model discussed here, which implies that the abundance of thiols and vicinal dithiols on the surface of mitochondrial proteins should be greater than expected by chance. Further, if surface thiols protect a particular protein from oxidative damage, then this can be assessed by replacing the relevant thiols.

Overview of mitochondrial thiol antioxidant defenses

Mitochondrial thiols are central to antioxidant defenses. A major way in which this occurs is by degrading peroxides through the Prx and Gpx systems operating in parallel (219). In addition, there are other enzymatic processes that sequester the reactive break-down products of lipid peroxidation and repair oxidized proteins within mitochondria. Finally, there is a high concentration of thiols on the surface of mitochondrial proteins, and these may be able to detoxify reactive species in conjunction with GSH/Grx2 and Trx2; however, the importance of these remain speculative.

Mediation of Redox Signaling by Mitochondrial Thiols

Redox signaling occurs when the function or activity of a biological system alters in response to a process associated with a change in the levels of a particular ROS or the reduction potential of a critical redox couple (38, 57, 93, 170). Thiol systems are often invoked as mediators or regulators of redox signaling in biological processes (51, 65, 66, 181). This is because thiols can be readily modified in response to changes in the reduction potential of a number of redox couples, or by the presence of a ROS such as hydrogen peroxide, and these alterations are generally easily reversed once the signal has diminished (9, 80, 91, 93). The general paradigm for how such redox signaling pathways work is illustrated in Figure 6. Here, it can be seen that a redox signal, such as hydrogen peroxide, a shift in the reduction potential of a linked redox couple, or the oxidation of an adaptor protein can lead to a reversible redox alteration to thiols on the target protein. Typically, these thiol modifications change the protein's function, such as its enzymatic activity, binding affinity to another protein, action as a transcription factor, or as a transporter or channel. Once the initial redox signal has returned to basal levels, the alteration reverses, and the protein's activity reverts to its initial level. This general scenario for redox signaling by thiol proteins has been found in a number of cytosolic signaling systems (6, 38, 49, 57, 145, 170, 171). However, there are also thiol signaling pathways in which the protein thiol modification is not reversed, as exemplified by the cytosolic NRF2-KEAP1 pathway in which thiols on the KEAP1 protein can react irreversibly with electrophiles to release the NRF2 transcription factor that translocates to the nucleus where it induces transcription of those genes under the control of promoters containing the antioxidant response element (78, 108).

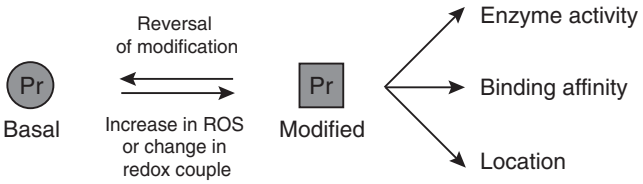

FIG. 6. Redox signaling by the mitochondrial protein thiol system. This shows how a reversible redox modification to a protein thiol can lead to the transmission of a redox signal.

Posttranslational modifications to protein thiols

Redox signals often impact on protein function by modifying an accessible thiol that changes its activity (38, 224). If the modification is to an active site thiol, for example, oxidation of the critical thiol in tyrosine phosphatases (13), then the impact on the protein is a clear loss of function. More generally, there are many other modifications that occur to thiols which are not essential for the activity of the protein that have less direct effects on protein function, and these are the most intriguing aspects of redox signaling. The alterations to protein thiols that can act as such posttranslational modifications include the formation of sulfenic acids (15, 23, 114), glutathionylation (33, 41, 60, 107, 138, 183, 184, 213, 224), intra- and interprotein disulfide formation (45), the formation of sulfenyl amides (186), and the formation of S-nitrosothiols (80, 82, 132, 190).

These posttranslational modifications can act as "redox switches" (19, 129, 181, 222), thus enabling the function of a protein to respond sensitively and reversibly to the reduction potential of a particular redox couple or to the production of a particular ROS. For example, the extent of glutathionylation on a particular mitochondrial protein thiol protein can be modified in response to changes in the GSH/GSSG ratio, mediated by Grx2 (8, 32, 181). Similarly, the redox state of the Trx2 pool, or of other dithiol proteins, can reversibly affect the activity of target proteins by introducing internal disulfides (45).

Thiol modifications can also occur independently of bulk changes in particular redox couples. For example, hydrogen peroxide can react with a thiol to convert it to a sulfenic acid, which can be a post-translational modification itself, or it could react with a GSH molecule to form a glutathionylated protein or with an adjacent thiol to form a disulfide (15, 23, 41, 89). Thus, the modification of the thiol by hydrogen peroxide can occur independently of changes in the GSH or Trx2 pools.

A related mode of protein thiol post-translational modification is the reversible formation of S-nitrosothiols, which is called S-nitrosation or S-nitrosylation (9, 80, 82, 188, 189). The mechanism of formation of S-nitrosothiols *in vivo* is obscure (82), but once formed on a protein or GSH, the S-nitrosothiol can be passed from thiol to thiol by transnitrosation, with the formation and stability of an SNO determined by protein sequence motifs around the modified cysteine residue (10, 48, 85, 132, 151). In addition, an initial S-nitrosothiol formed on a protein may go on to form other modifications, such as by a reaction with a thiol to displace NO^- and thereby generate a disulfide or a glutathionylated protein (151, 190).

To be effective redox signals, these post-translational modifications have to affect the function of the protein and be reversible so that the modification returns to baseline levels once the signal has expired. Often redox signaling is compared, explicitly or tacitly, to signaling by reversible protein phosphorylation. In phosphorylation, there is a large thermodynamic driving force for the phosphorylation of serine, threonine, or tyrosine residues that is channeled and kinetically controlled by tightly regulated kinases. The site and stability of the phosphorylation is strongly influenced by local sequence and structural motifs. The introduction of a bulky, charged phosphate group has a significant effect on the activity or binding of the target protein, thus resulting in a change in its function or location. The reversal of the modification is also tightly regulated by specific phosphatases. Very few redox signaling pathways are as well defined as this, with most only matching a few aspects of the phosphorylation paradigm. Often the processes that lead to the redox modifications are nonspecific with selectivity determined by the environment of the thiol that is modified. Structural alterations brought about by glutathionylation, disulfide formation, or S-nitrosation can potentially have a major effect on protein function, but in only a few cases have detailed structural analyses shown clearly how this occurs. Finally, in contrast to the action of phosphatases, many processes that can reverse redox modifications are relatively nonspecific. However, some reactions such as the deglutathionyaltion of a gluathionylated protein by Grx may be specific (61, 72), and, thus, the lifetime and reversal of a particular alteration can be determined by the environment of the thiol.

Reversible thiol modifications in mitochondrial redox signaling

A considerable body of evidence has been built up that demonstrates the presence of redox modifiable protein thiols within mitochondria (29, 86–88, 163, 192). When redox proteomic techniques are used, a range of proteins have been identified that contain reversibly modified thiols which respond to exogenous hydrogen peroxide, NO-donors, and thiol oxidants such as diamide (88). More specialized approaches further enable the identification of proteins that contain particular thiol modifications such as S-nitrosation (29, 39, 196) or glutathionylation (8, 88). These proteomic approaches have been extended to *in vivo* situations, and a range of mitochondrial proteins have been found to have reversible modifications *in vivo* (17, 23, 48, 59, 146, 147, 182, 193). The individual proteins affected and the physiological implications of these modifications are beyond the scope of this article. Even so, it is clear that there is a pool of readily modifiable protein thiols within mitochondria which are altered by mild oxidative and nitrosative signals.

Since many of the proteins that are susceptible to redox modification are central to metabolism, it is tempting to view this as evidence of redox regulation of mitochondrial function. However, before this can be done, it is important to establish that the protein's activity is affected by the modification and that this change in activity returns to baseline when the modification is reversed. In a few cases, it has been shown that a modification such as S-nitrosation decreases the activity of a mitochondrial protein and that this activity loss was reversed by the removal of the modification (29, 88, 192, 193). It is also important to show that the extent of the modification correlates with the change in activity, the cysteine residues affected have to be identified, and, ideally, the effect of replacing the responsive cysteine residue should be determined and be shown convincingly to be biologically important.

Changes in ROS levels in response to thiol oxidation

Changes in the redox state of the mitochondrial protein thiols can modulate the levels of redox signaling molecules such as hydrogen peroxide (225). Hydrogen peroxide is generated in response to a range of signaling pathways by NADPH oxidase enzymes at the cell surface and then goes on to modify various proteins within the cell (93, 173, 174). There are indications that this signal can be relayed to mitochondria where it can modulate metabolic processes; mitochondria also produce hydrogen peroxide and can, thus, act as a source of this redox signal to the rest of the cell (145). Consequently, the extent of degradation of hydrogen peroxide within mitochondria can impact on redox signaling to, within, and through mitochondria (145). The activity of mitochondrial peroxidases, notably Prx3, is altered by its exposure to hydrogen peroxide, and this may affect the release of hydrogen peroxide from the mitochondria (36). This change in activity in the major mitochondrial peroxidase will, in turn, lead on to the changes in the level of hydrogen peroxide within the mitochondria and in the amounts emanating from the organelles and will thereby have a major impact on redox signaling pathways (34–36, 173, 174). This is supported by the finding that the oxidation of protein thiols increases hydrogen peroxide efflux from isolated mitochondria (109, 175) (Fig. 7), and may contribute to the phenomenon of ROS induced ROS release, where previous exposure to ROS affects subsequent ROS release (225). There is also the possibility that the activity of mitochondrial Prxs can be regulated by phosphorylation or by oligomerisation of the protein, but whether this occurs to regulate hydrogen peroxide production within mitochondria is currently unclear. Thus, it remains an intriguing possibility that modification of the activity of mitochondrial peroxidases may be an important way of modulating the levels of signaling ROS such as hydrogen peroxide within mitochondria and the rest of the cell. Superoxide can also diffuse from mitochondria through ion channels in the inner membrane and may also act as a redox signal, and the activity of these channels may be modified in response to thiol oxidation (223, 225).

It is also possible that thiol modifications can change the rate of production of ROS within mitochondria through post-translational modification of components of the mitochondrial respiratory chain and of other enzymes within

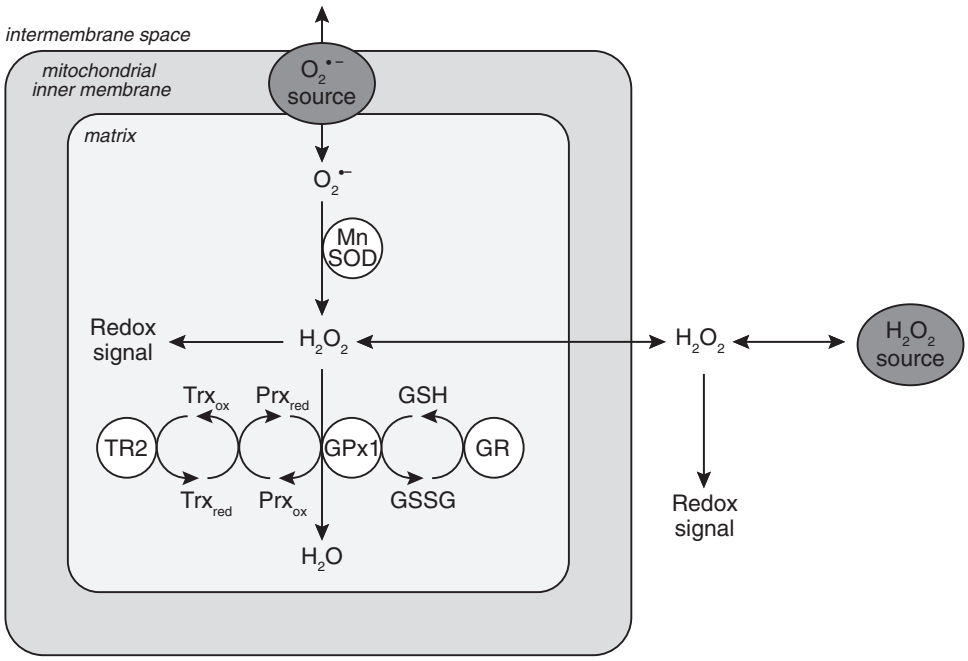

FIG. 7. Modulation of hydrogen peroxide signaling by the mitochondrial protein thiol system. Hydrogen peroxide can be generated as a redox signal from superoxide production by the mitochondrial respiratory chain or from the action of cell surface oxidases. The levels of hydrogen peroxide within mitochondria and that are released from mitochondria are altered by the action of mitochondrial peroxidases.

mitochondria (145). This may occur by either reversibly modifying the activity of the protein and thereby altering superoxide production by these enzymes (24, 26, 145).

Alternatively, disrupting the activity of these proteins by irreversible inhibition with a thiol reactive species such as 4-hydroxynonenal (47, 117) or glyoxal (218) may alter mitochondrial superoxide production. Theses modification could act on the protein itself to increase superoxide leak or it could inhibit respiration and lead to a consequent build up of electrons on the NADH or coenzyme Q pools and that, in turn, can lead to increased superoxide production at various sites in the mitochondrion (145).

The Overlapping Roles of Mitochondrial Thiols

The thiol systems used in protection against oxidative stress and in redox signaling are essentially the same. Thus, protection and signaling are not separate processes but are both aspects of the interactions of mitochondrial thiols with reactive species and redox challenges. Consequently, there is considerable overlap between the signaling and protective effects of mitochondrial thiol alterations.

A further consideration is that it is frequently unclear what is meant by redox signaling, as often the tacit assumption is that a signaling pathway should be similar to the classic phosphorylation signaling pathways with regulated on/off mechanisms and a major change in protein function on post-translational modification. In some cases, it is as if the redox signaling field suffers from "phosphorylation envy" with all thiol changes to proteins being interpreted as if they were vital components of a signaling pathway. However, many redox changes to protein thiols are less specific than required for a signaling cascade, and many redox alterations occur without having a clear functional role. It is more likely that there is a continuum of redox changes to protein thiols with only a small number being classic redox signaling pathways or vital for protection with the majority having minor effects on the activity of a particular pathway or on protection against oxidative damage.

The concept that many redox changes assist the response of a metabolic system to changes in the redox milieu without being vital for defense or signaling has been introduced by Jones and referred to as "redox sensing" (97). This framework is a useful way to interpret the thiol redox changes wrought on mitochondria by redox challenges suggesting that the majority of thiol changes are orthogonal to the few major pathways of defense and signaling (97). In other words, although redox changes may be a major component of some signaling pathways, the majority are likely to be relatively minor modifications of other pathways; so, the redox changes are distinct from, or orthogonal to, the existing regulatory frameworks. Even so, these orthogonal thiol modifications still allow the system to sense and respond to redox

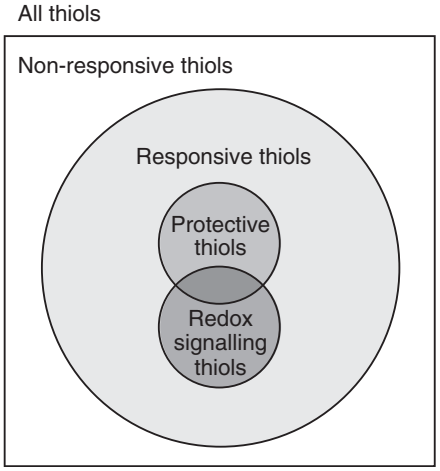

FIG. 8. Venn diagram of overlapping roles of mitochondrial protein thiols.

alterations with integrated changes in function. Despite the limitations due to the inevitable imprecision in concepts such as redox sensing, this is heuristically useful as a way to proceed and will be modified as more precise information on the roles and changes to individual mitochondrial thiols is acquired over time. This view of mitochondrial thiols is illustrated in a Venn diagram which distinguishes between those thiols involved in regulation, those essential for protection, those that contribute to redox sensing and the overall response to oxidative challenges, and those that are inactive (Fig. 8). Although this outline is speculative, it helps illustrate the many overlapping roles of thiols within the mitochondrial matrix and how this integrated system enables mitochondria to respond to and protect itself from oxidative damage and redox challenge by modulating the function of mitochondrial thiol systems.

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Abbreviations Used

Gpx = glutathione peroxidases
GR = glutathione reductase
Grx = glutaredoxin
GSH = glutathione
GSSG = glutathione disulfide
GST = glutathione-S-transferases
ICDH = isocitrate dehydrogenase
MSR = methionine sulfoxide reductase
MnSOD = manganese superoxide dismutase
NMN = nicotinamide mononucleotide
$NO^{\bullet}$ = nitric oxide
Prx = peroxiredoxin
ROS = reactive oxygen species
SNO = S-nitrosothiol
Srx = sulfiredoxin
TCA = tricarboxylic acid
TH = transhydrogenase
TR2 = thioredoxin reductase
Trx = thioredoxin

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