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Forum Review

Glutathionylation of Mitochondrial Proteins

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ABSTRACT

Many proteins contain free thiols that can be modified by the reversible formation of mixed disulfides with low-molecular-weight thiols through a process called *S*-thiolation. As the majority of these modifications result from the interaction of protein thiols with the endogenous glutathione pool, protein glutathionylation is the predominant alteration. Protein glutathionylation is of significance both for defense against oxidative damage and in redox signaling. As mitochondria are at the heart of both oxidative damage and redox signaling within the cell, the glutathionylation of mitochondrial proteins is of particular importance. Here we review the mechanisms and physiological significance of the glutathionylation of mitochondrial thiol proteins. *Antioxid. Redox Signal.* 7, 999–1010.

INTRODUCTION

PROTEIN *S*-THIOLATION is the formation of mixed disulfides between a protein thiol and a small-molecular-weight thiol (38–40, 94). As glutathione (GSH) is the dominant low-molecular-weight thiol in the cell, it is the main molecule linked to protein thiols by a disulfide bond and, therefore, we have focused on *S*-glutathionylation. The reversible glutathionylation of proteins is important in the response of cells to oxidative damage and also in redox signaling (38–40, 94). As mitochondria are central to cellular oxidative damage and redox signaling, the glutathionylation of mitochondrial proteins is particularly important. Here we review the mechanisms by which mitochondrial protein thiols can be glutathionylated and discuss the potential physiological and pathological significance of these processes.

MITOCHONDRIAL PROTEIN THIOLS AND GLUTATHIONE

The critical interaction for protein glutathionylation is that between protein thiols and glutathione (20, 50, 94). There is a high concentration of both protein thiols and glutathione within mitochondria, in part to protect mitochondria against reactive oxygen species (ROS), and these thiols are an important component of the cell's antioxidant defenses (72, 89). Mitochondria have a comprehensive array of interacting thiol-metabolizing systems that can be categorized as protein thiols, low-molecular-weight thiols (predominantly glutathione), and the enzymes that act on them (19). Although a major function of these thiol systems is to protect mitochondria from oxidative stress, they may also be involved in the regulation of mitochondrial function.

Protein thiols

There is a range of mitochondrial protein thiols that can be broadly divided into essential thiols in the active sites of enzymes, thiols exposed to the aqueous environment on the surface of proteins, and buried protein thiols that may play a structural role (for example, in iron-sulfur centers or zinc finger motifs). It is the exposed thiols that are of interest as these can interact with the mitochondrial GSH pool. These reactive protein thiols include regulatory protein thiols, alterations to which modulate protein function, and thiols involved in antioxidant defense. Within mitochondria, these exposed, surface protein thiols are present at a high concentration. For example, in bovine heart mitochondrial membranes, there are ~35 nmol of exposed thiols/mg of protein comprising ~40% of the total membrane protein thiols present (6). Similarly, in intact rat liver mitochondria, there are ~65–70 nmol of thiol/mg of protein in total, and ~20–25 nmol of these are ex-

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posed and reactive in native mitochondria (66). This contrasts with a glutathione content for isolated liver mitochondria of 3–5 nmol/mg of protein (66, 93). Thus, the thiol concentration within the mitochondrial matrix due to exposed protein thiols is greater than that of GSH, suggesting that the exposed protein thiols and their interactions with the GSH pool play an important role in mitochondrial thiol metabolism.

The mitochondrial glutathione pool

GSH is a small, hydrophilic molecule formed from the amino acids glycine, cysteine, and glutamate. It is present in high concentrations within the cytosol and mitochondria as the predominant low-molecular-weight thiol (28, 72, 89, 94). The concentration of GSH within mitochondria is 5–10 m*M*, about the same as in the cytosol (47, 54, 73, 89), although there is a recent indication that the mitochondrial glutathione concentration may be higher than in the cytosol and that there may also be variations in glutathione content between mitochondria (97).

Glutathione does not originate in mitochondria, but is synthesized in the cytoplasm and then imported (28, 47). The separation of the mitochondrial and cytosolic glutathione pools was demonstrated by the biphasic decline of glutathione in isolated hepatocytes, and in the liver *in vivo,* on administration of the glutathione synthesis inhibitor, buthionine sulfoximine (BSO) (47, 73). Cytosolic glutathione in isolated hepatocytes was depleted relatively rapidly ($t_{1/2}$ = 2 h) by BSO, whereas the mitochondrial pool was lost far more slowly ($t_{1/2}$ = 30 h), suggesting that mitochondria can maintain their glutathione pool even when that in the cytosol has been depleted or oxidized (47, 70, 72, 73). A further implication of these findings is that the mitochondrial and cytoplasmic glutathione pools can change their redox states independently. Consequently, the mitochondrial GSH/glutathione disulfide (GSSG) ratio can vary independently of that of the cytosol (73), and such changes are likely to contribute to the response of mitochondria to redox signals and oxidative damage.

Even so, the mitochondrial and cytosolic glutathione pools must communicate as glutathione is imported into mitochondria from the cytosol (28, 47). Furthermore, there is rapid exchange of cytosolic and mitochondrial GSH, confirmed by the rapid equilibration of the specific activity of the two pools when the cytosolic pool is "spiked" with [^{35}S]cysteine (28, 47, 72). This suggests that there are glutathione transporters in the mitochondrial inner membrane that catalyze rapid GSH/GSH exchange, but not the net transport of glutathione equivalents from mitochondria to the cytoplasm (28, 47). The nature of the glutathione transporter(s) in the mitochondrial inner membrane is uncertain, but there does not appear to be a specific mitochondrial GSSG efflux system (81). There was, however, rapid membrane potential-dependent uptake of GSH by isolated liver mitochondria by both high- and low-affinity transport systems (47, 62, 70, 73). In contrast, isolated kidney mitochondria had only a low-affinity uptake system that was not membrane potential-dependent, and in this case uptake was by electroneutral exchange with other dicarboxylates (71). A further contrast to liver mitochondria was that kidney mitochondria took up GSSG, although the low GSSG affinity of this process makes a physiological role unlikely (28, 47). The efflux of GSH from GSH-loaded liver mitochondria was stimulated by external GSH, although at higher external GSH concentrations there was reuptake of the released GSH, implying sequestration of GSH in the intermembrane space (70, 72). A GSH transport activity was isolated and reconstituted into liposomes by techniques that are selective for the mitochondrial carrier superfamily (15, 28, 47). However, these putative mitochondrial glutathione carrier(s) have not been identified definitively, and the uptake mechanisms may be tissue-specific (15, 28, 71).

Reactions of the mitochondrial glutathione pool

The mitochondrial glutathione pool is an important means of protection against oxidative damage, both by direct reaction with ROS or reactive nitrogen species (RNS) and as an electron donor for antioxidant enzymes (72, 89) (Fig. 1). GSH also protects against toxins through glutathione *S*-transferases, which convert electrophilic xenobiotics and lipid peroxidation products, such as 4-hydroxynonenal, to thioethers for excretion (34, 86). GSH is oxidized to GSSG by the direct reaction of the thiol of GSH with ROS, or through catalysis by glutathione peroxidases that reduce hydrogen peroxide (H_2O_2) and alkyl peroxides (ROOH) using GSH as the elec-

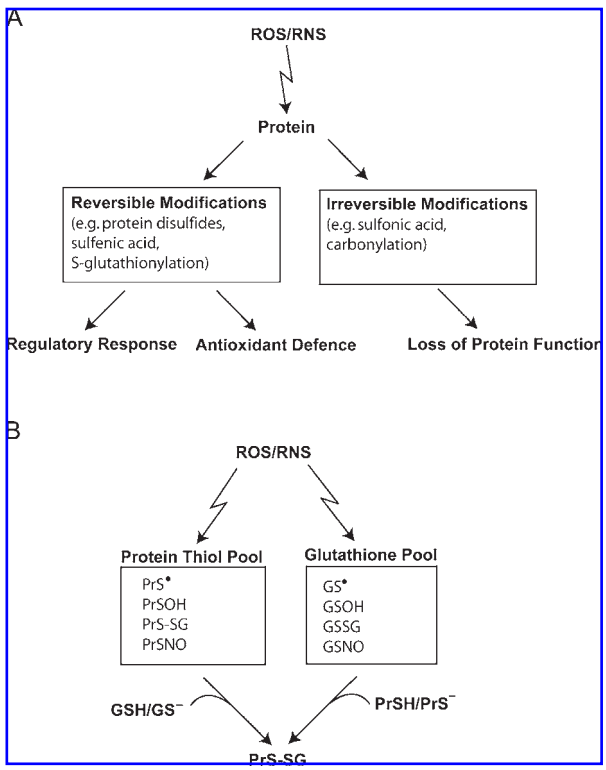


FIG. 1. Interaction of the mitochondrial thiol system with ROS and RNS. (**A**) The general interactions of mitochondrial protein thiols with ROS/RNS. (**B**) The ways in which mitochondrial thiol proteins can become glutathionylated.

tron donor (26, 50, 84, 94). Mitochondria have a soluble glutathione peroxidase (Gpx1), which is a 22-kDa selenoenzyme that is highly expressed in mitochondria from the liver and kidney, but poorly expressed in heart and muscle (26, 84). In addition to H_2O_2, phospholipid hydroperoxides (PlOOH) are a common consequence of oxidative damage, particularly to phospholipids such as cardiolipin, which have a high proportion of unsaturated fatty acids (21). To degrade PlOOH, there is a specific phospholipid hydroperoxide glutathione peroxidase (PHGPx), a selenoenzyme that directly reduces lipid hydroperoxides to water and a hydroxylated lipid (1). There is a short isoform of this enzyme present in the cytoplasm and a longer, mitochondrial isoform that has an N-terminal targeting peptide that is processed to give a mature membrane-bound enzyme of 20 kDa (1, 44).

The reactions described lead to the conversion of GSH to GSSG; consequently, glutathione reductase (GR) plays an essential role in continually recycling GSSG back to GSH (19, 47, 89). A single gene encodes both cytoplasmic and mitochondrial isoforms of GR through alternative transcription initiation sites (104, 105). The mature mitochondrial GR is a homodimer of 51.7-kDa monomers (104, 105). Mitochondrial GR is a flavoenzyme that uses NADPH as the electron donor to reduce GSSG to GSH (59, 80, 104, 105). To maintain sufficient NADPH to supply GR, the mitochondrial NADPH/NADP ratio is kept high by a NADP-dependent isocitrate dehydrogenase (53), and by a transhydrogenase that utilizes the proton electrochemical potential difference across the inner membrane to drive electrons from NADH to NADP (8). Through these reactions, the ratio of GSH to GSSG in mitochondria is kept high and the mitochondrial glutathione pool is typically 95–99% reduced, except during oxidative stress (89, 94).

PROTEIN GLUTATHIONYLATION

Protein thiols can interact with the mitochondrial glutathione pool by a number of mechanisms that lead to their glutathionylation.

Thiol–disulfide exchange

A major pathway for protein glutathionylation is thiol–disulfide exchange between a free protein thiol and GSSG. The attacking nucleophile is the thiolate anion of the protein thiol, which reacts with GSSG to form a mixed disulfide, releasing glutathione (Reaction 1). Due to the requirement of the thiolate anion for this reaction, (pK_a typically 8.5–9), thiol reactivity is particularly sensitive to pH in the physiological range. As the mitochondrial matrix is at pH 8, thiol–disulfide exchange is facilitated within mitochondria.

$$PrS^- + GSSG \longrightarrow PrS\text{-}SG + GS^-$$

Reaction 1

The high intramitochondrial GSH/GSSG ratio minimizes protein glutathionylation by thiol–disulfide exchange (50, 94). However, during oxidative stress or redox signaling, ROS oxidize GSH to GSSG either directly or through the catalytic action of glutathione peroxidases. Protein thiols can then respond to the decreased GSH/GSSG ratio by forming mixed disulfides with glutathione through thiol–disulfide exchange between the thiolate anion and GSSG. The reactivity of protein thiols with GSSG depends on the pK_a of a given thiol and on the accessibility of the thiol to GSSG, both of which can vary widely. The pK_a of protein thiols can be altered dramatically depending on the local environment of the cysteine residue (23, 38), for example, in the active-site thiols of cysteine proteases (57, 77). This variability in thiol reactivity is important for exposed thiols as it enables them to respond by altering their glutathionylation status in a graded fashion to changes in the GSH/GSSG ratio, with implications for both the antioxidant and redox signaling roles of protein thiols.

Thiol oxidation

Another cause of protein glutathionylation is oxidative damage to the protein thiol. One such pathway to mixed disulfides is through the formation of a thiyl radical ($PrS^{\bullet}$) by the one-electron oxidation of a protein thiol (56, 109). The thiyl radical can then react with a glutathionylate anion (GS^-) to form a radical mixed disulfide ($PrS^{\bullet-}$-SG), which will lose an electron to oxygen to form superoxide ($O_2^{\bullet-}$) leaving a mixed disulfide (113) (Reaction 2).

$$PrS^{\bullet} + GS^- \longrightarrow PrS^{\bullet-}\text{-}SG \longrightarrow PrS\text{-}SG$$

$$H^+ \qquad O_2 \qquad O_2^{\bullet-}$$

Reaction 2

Another route to mixed disulfides by oxidative damage is through the two-electron oxidation of a thiol to a sulfenic acid (RSOH), which will then react with a glutathionylate anion to displace OH^- and generate a disulfide (94) (Reaction 3).

$$PrSOH + GS^- \longrightarrow PrS\text{-}SG + OH^-$$

Reaction 3

The reactions of protein thiyl radicals and sulfenic acids with GSH to form mixed disulfides (Reactions 2 and 3) are important antioxidant defenses as they prevent further irreversible protein oxidation. Furthermore, as the two-electron oxidation of thiols to sulfenic acids is a common result of the reaction of peroxides or peroxynitrite with thiols, this may give these molecules the opportunity to act as signals by altering protein glutathionylation status. Finally, the reactions of ROS with protein thiols could just as easily occur with the thiols of GSH, and if GSH is converted to a thiyl radical or a sulfenic acid, it too will react with a protein thiol to form a glutathionylated protein by a similar mechanism to Reaction 2 or 3.

Glutathionylation induced by nitric oxide (NO)

The interaction of NO with GSH or thiols may also lead to the formation of glutathionylated proteins. Mitochondria are exposed to excess NO under a range of pathological conditions (9, 76), and electron transport may also be regulated by the interaction of NO with cytochrome oxidase (11, 17, 75).

The presence of a putative mitochondrial NO synthase is also evidence for a possible physiological role for NO within mitochondria (25, 36, 58, 92, 107). This exposure of mitochondria to NO can lead to the formation of peroxynitrite, which can oxidize thiols to either thiyl radicals or sulfenic acids and lead to protein glutathionylation by the mechanisms shown in Reactions 2 and 3 above (4, 9, 88). In addition, there is considerable interest in the formation of *S*-nitrosylated proteins on exposure to NO and whether this acts as a regulatory mechanism (30, 100, 101). It is possible that *S*-nitrosylation of a protein thiol to form a PrSNO can lead to protein glutathionylation. This might occur by the displacement of the nitroxyl anion (NO^-) by the glutathionylate anion to form a glutathionylated protein (10, 30, 100, 101) (Reaction 4).

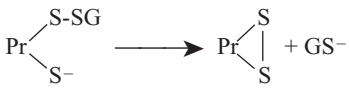
$$PrSNO + GS^- \longrightarrow PrS\text{-}SG + NO^-$$

Reaction 4

The formation of *S*-nitrosylated GSH (GSNO) is a common consequence of exposure to NO and nitrosative stress (103). GSNO may react with protein thiols to displace NO^- and generate a glutathionylated protein by an analogous mechanism to Reaction 4 (60, 82, 83), although the competing transnitrosation reaction in which NO^+ is moved from one thiolate to another may also occur. The relative contributions of these reactions to protein glutathionylation *in vivo* are uncertain, and the interpretation of experiments is often complicated by the breakdown of GSNO to GSSG. However, the possibility that NO can prime a protein thiol for subsequent glutathionylation is an intriguing one that would allow the glutathionylation status of a particular protein thiol to be altered independently of changes in the bulk GSH/GSSG ratio.

FATE OF GLUTATHIONYLATED PROTEINS

The mechanisms described above can all lead to the formation of a protein-glutathione mixed disulfide on a protein. Although the disulfide bond linking the protein and the glutathione is readily reversible under reducing conditions, under oxidizing conditions the glutathionylation can be maintained indefinitely as a persistently glutathionylated protein (6, 38). However, in many situations the glutathionylation is only transient as an adjacent protein thiol displaces the GSH to form an intraprotein disulfide (Reaction 5) (38).



Reaction 5

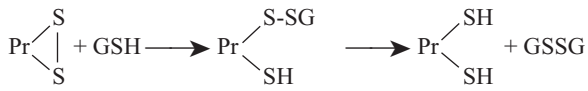
Thus, there are two classes of glutathionylated proteins, those that are transiently glutathionylated before protein disulfide formation, and those that are persistently glutathionylated, of which the former is more common (6). Consequently, on oxidizing protein thiols the proportion that formed persistently glutathionylated proteins was far smaller than that which formed intraprotein disulfides (6). The mechanistic reason for the tendency toward intraprotein disulfide formation is presumably that glutathionylation normally occurs on protein thiols that are adjacent to a second thiol that rapidly displaces GSH to form an internal disulfide (Reaction 5). This juxtaposition could arise by chance; however, there are reasons related to both redox regulation and antioxidant defense to favor the formation of intraprotein disulfides over mixed disulfides, and these are discussed later on. In addition, Reaction 5 could also occur between adjacent proteins leading to an interprotein disulfide link, although the evidence from mitochondrial membrane studies suggests that most of the disulfides formed are intraprotein (6).

Once the GSH/GSSG ratio has returned to its resting state due to the action of GR, the high GSH/GSSG ratio will lead to reversal of the glutathionylation by thiol–disulfide exchange (Reaction 6).

$$PrS\text{-}SG + GSH \longrightarrow PrSH + GSSG$$

Reaction 6

If the protein has formed an internal disulfide, then thiol–disulfide exchange with GSH will lead to the reduction of the disulfide with the transient formation of a glutathionylated intermediate.



Reaction 7

The reversal of glutathionylation and the reduction of intraprotein disulfides can also be catalyzed by enzymes such as glutaredoxin (Grx) and thioredoxin (Trx).

GLUTAREDOXIN

For both regulatory and antioxidant roles, it is important for the protein thiol redox state to respond rapidly to changes in the GSH/GSSG ratio. Thiol–disulfide exchange between GSSG and a protein thiol (Reaction 1) and the reverse reaction between a glutathionylated protein and GSH (Reaction 6) are often relatively slow (23, 38, 115). Therefore, these reactions are catalyzed by the small, soluble protein Grx (3, 46, 91). Grx from *Escherichia coli* has a CPYC active-site motif with a solvent-exposed Cys^{11}, whereas Cys^{14} is buried within the enzyme. Both Cys residues are required for the direct reduction of protein disulfides by Grx, and the disulfide form of Grx is reduced back to the dithiol by reaction with GSH (12). Only Cys^{11} is necessary for glutathionylation/deglutathionylation, facilitated by the adjacent glutathione binding site and by its very low pK_a (46). The Grx-SG mixed disulfide intermediate is reduced to a dithiol by GSH (12, 46, 114). Grx catalyzes the deglutathionylation of protein-glutathione mixed disulfides (Reaction 6) far more effectively than Trx or protein disulfide isomerase (56). Grx can also act as an antioxidant by directly scavenging glutathionyl radicals (102).

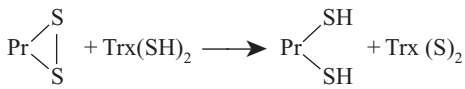
Recently, a mitochondrial isoform, Grx2, has been discovered that has an N-terminal mitochondrial targeting peptide that, on processing, yields a mature protein of ~15 kDa (43, 67). Mammalian Grx2 has a CSYC active-site motif, instead of the CPYC motif of the mammalian cytosolic and *E. coli*

enzymes, with Cys^{70} being critical for glutathionylation, similar to Cys^{11} in *E. coli* (55). Modeling suggests that the GSH binding site and the hydrophobic surface of Grx2 are similar to those of Grx1 (43, 67). However, there are significant differences between the two isoforms: Grx2 lacks one of the conserved non–active-site Cys residues of Grx1 (67) and is consequently less easily inactivated by oxidants and GSSG (43); in addition, Grx2 can be reactivated directly by thioredoxin reductase (TrxR) as well as by GSH (55). These differences may help Grx2 to operate in the more oxidatively stressed mitochondrial environment (55).

Grx2 has been shown to catalyze effectively both the glutathionylation and deglutathionylation of mitochondrial thiol proteins and the reduction of protein disulfides (6, 55). It is likely to be an essential component of the mitochondrial antioxidant defenses: in support of this, selectively decreasing Grx2 levels by RNAi led to increased sensitivity to oxidative damage in HeLa cells (65). The rapid deglutathionylation of mixed disulfides by Grx2 will quickly restore the protein thiols and supports a role for exposed protein thiols in mitochondrial antioxidant defense in which protein thiols detoxify ROS forming protein thiyl radicals and sulfenic acids (Reactions 2 and 3). These are then recycled back to protein thiols by Reaction 6. Interestingly, we showed that the oxidation and persistent glutathionylation of protein thiols by GSSG were catalyzed dramatically by Grx2, even at relatively reduced GSH/GSSG ratios (6), indicating that Grx2 could contribute to regulatory changes in protein glutathionylation. Thus, Grx2 stands at the center of the reversible interactions of protein thiols with the mitochondrial glutathione pool (Fig. 2).

THIOREDOXIN

There are other enzyme systems in addition to Grx that interact with protein-glutathione mixed disulfides and internal protein disulfides, the most important of which is the Trx/TrxR system (49). Trx are small thiol proteins that have an active-site dithiol that reduces protein disulfides, leaving an internal disulfide on Trx (2, 49). The disulfide form of Trx is reduced to its active dithiol by NADPH, catalyzed by TrxR (49). Mitochondria contain their own Trx (Trx2), which is a 12.2-kDa mature protein (98, 106), and their own TrxR (TrxR2), which is a homodimer of 55-kDa subunits, that is abundant in heart, liver, kidney, and adrenal gland mitochondria (35, 63, 74, 112). TrxR2 is an FAD-containing selenoenzyme that uses matrix NADPH to reduce the disulfide form of Trx2 (35, 74, 112). As well as reducing protein disulfides, Trx can also remove glutathione from glutathionylated proteins, although with markedly different rates of deglutathionylation for different protein mixed disulfides and at a lower rate than Grx (56, 85). Therefore, the major function of Trx2/TrxR2 in mitochondria is likely to be the direct reduction of intraprotein disulfides (2, 49) (Reaction 8), although the direct reduction of Grx2 by TrxR2 may also be important under some circumstances (55).



Reaction 8

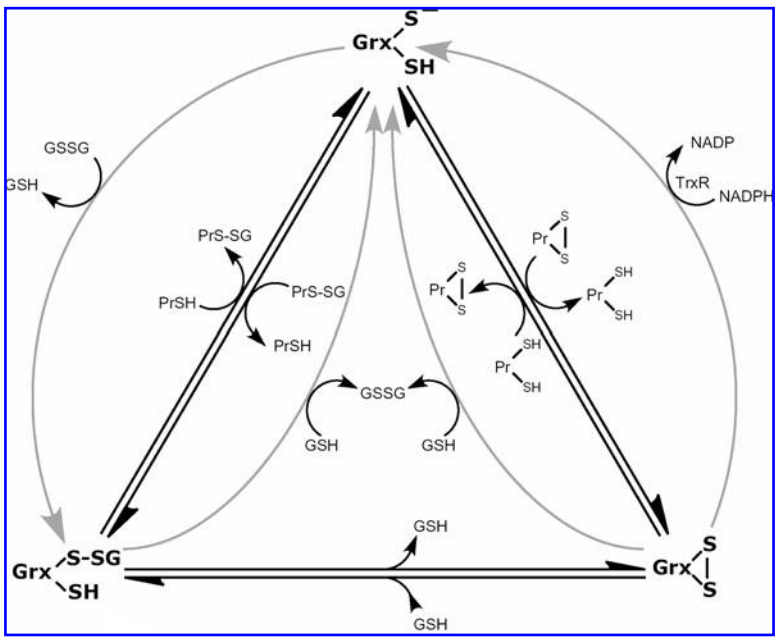


FIG. 2. Grx2 catalyzes reversible protein thiol glutathionylation and disulfide formation. This scheme shows how Grx2 interconverts between the dithiol, glutathionylated, and disulfide forms. In doing so, it catalyzes the glutathionylation and deglutathionylation of protein thiols and the reversible formation of protein disulfides. Grx, glutaredoxin 2; GSH, glutathione; GSSG, glutathione disulfide; PrSH, protein thiol; PrS-SG, protein-glutathione mixed disulfide; TrxR, thioredoxin reductase. The black lines are the principal reactions of Grx with proteins, whereas the gray lines are its reactions with GSH, GSSG, and TrxR.

PHYSIOLOGICAL ROLES OF MITOCHONDRIAL PROTEIN GLUTATHIONYLATION

The reversible glutathionylation of protein thiols occurs by the mechanisms outlined above and is thought to have two major biological functions within mitochondria: as part of the organelle's antioxidant defense strategies and as a component of a redox signaling pathway. It is sometimes difficult to determine if a particular reaction is an antioxidant or a signaling reaction, and of course many reactions will be both.

PROTEIN GLUTATHIONYLATION AS AN ANTIOXIDANT DEFENSE

There are several ways in which the interaction of the glutathione pool with protein thiols can be an antioxidant defense. As the concentration of exposed reactive protein thiols in mitochondria is greater than that of GSH, the direct reaction of protein thiols with ROS may be important for antioxidant defenses (56, 94, 109). Reactions with ROS convert protein thiols to thiyl radicals or sulfenic acids, which can be further oxidized by direct reaction with oxygen to form higher oxidation states, such as sulfinic (RSO_2H) and sulfonic (RSO_3H) acids (56, 94, 109), which are poorly repaired. The rapid reactions of protein thiyl radicals or sulfenic acids with GSH therefore have important antioxidant roles because they prevent the formation of these higher oxidation states. This reaction with GSH to form a radical mixed disulfide may be a major pathway for the repair of thiyl radicals *in vivo*, diverting the radical to superoxide, which can then be detoxified by superoxide dismutase (113). The protein mixed disulfide can then be reduced back to a protein thiol by GSH (Reaction 6). Therefore, this reaction can facilitate the antioxidant role of protein thiols.

Another putative antioxidant role for the formation of protein mixed disulfides within mitochondria is to buffer the GSH/GSSG ratio, by reacting with GSSG to release one or two GSH molecules, leaving a protein mixed disulfide (Reaction 1) or a protein disulfide (reverse of Reaction 7) (109). This will help minimize oxidation of the glutathione pool during transient oxidative stress (94, 109). Once the oxidative stress has subsided, the protein mixed disulfide or intraprotein disulfide will be reduced back to a protein thiol by GSH (Reactions 6 and 7), Trx2 (98) (Reaction 8), or Grx2 (Fig. 2) (43, 67). However, the significance of this process *in vivo* is uncertain as the high concentration of protein thiols relative to GSH suggests that the direct reaction of protein thiols may be the predominant antioxidant defense, and it is unclear if it is the protein thiols that are buffering the GSH or vice versa. However, as a high GSH/GSSG ratio and a high GSH concentration are required for the action of glutathione peroxidases and of glutathione *S*-transferases, then it is likely that the maintenance of a high GSH/GSSG ratio through buffering by protein thiols does contribute to antioxidant defenses.

Protein glutathionylation as a regulatory response

In addition to antioxidant functions, there is growing evidence of a role for protein glutathionylation in redox sensing and signaling (20, 31, 39, 40, 42, 94, 109). Both glutathionylation and the formation of intraprotein disulfides can dramatically affect the activity of enzymes, transcription factors, and transporters, enabling them to respond reversibly to the ambient GSH/GSSG ratio, just as proteins are regulated by reversible phosphorylation (14, 20, 31, 39, 40, 94, 109, 114). Supporting such a role, a large number of enzymes and proteins undergo alterations in activity on glutathionylation or the formation of an intraprotein disulfide (20, 31, 109). However, one significant difference between this process and phosphorylation is that as glutathionylation can occur by thiol–disulfide exchange between protein thiols and the bulk glutathione pool, it is less selective than residue-specific phosphorylation and dephosphorylation by kinases and phosphatases. Even so, the selectivity for glutathionylation of a particular protein thiol may be strongly influenced by the effect of surrounding amino acid residues on its pK_a and accessibility. In addition, protein thiol glutathionylation may arise on particular protein thiols following their transformation into *S*-nitrosothiols (Reaction 4), or sulfenic acids (Reaction 3) formed from reaction with H_2O_2 or peroxynitrite. Furthermore, glutathionylation and deglutathionylation can be catalyzed by Grx, which may help to form or degrade mixed disulfides at particular protein thiols and thus modulate the lifetime of critical protein-glutathione mixed disulfides (56, 61, 85). Although these suggestions are speculative, a corollary of this is that protein glutathionylation may be both a general response to oxidative stress with bulk changes to most exposed protein thiols (20, 109, 115), and also a method of selectively modulating a small group of critical regulatory thiols. In support of this, it has been shown that there are different classes of protein thiols with a range of propensities to form protein mixed disulfides within cells under oxidative stress (96). Furthermore, there does seem to be a level of basal protein glutathionylation in resting cells despite a fully reduced GSH pool (31, 32, 42).

Protein activity can change following formation of an intraprotein disulfide, as happens for the transcription factor OxyR (114), or by formation of a protein-glutathione mixed disulfide, as occurs with carbonic anhydrase (68). However, for these changes in protein activity to function as redox switches in response to the GSH/GSSG ratio, the ratio of oxidatively modified to unmodified protein must change appropriately (39, 40, 94). For the formation of a protein mixed disulfide (PrS-SG), the equilibrium is:

$$K_1 = [\text{PrS-SG}][\text{GSH}]/[\text{PrSH}][\text{GSSG}] \qquad \text{(Eq. 1)}$$

In contrast, for the formation of an intraprotein disulfide (PrS_2) the equilibrium is:

$$K_2 = [\text{PrS}_2][\text{GSH}]^2/[\text{PrSH}][\text{GSSG}] \qquad \text{(Eq. 2)}$$

Hence, the PrSH/PrS_2 ratio is proportional to $[GSH]^2$, whereas the PrSH/PrSSG ratio is proportional to [GSH].

$$[\text{PrS-SG}]/[\text{PrSH}] = K_1\,[\text{GSSG}]/[\text{GSH}] \qquad \text{(Eq. 3)}$$

$$[\text{PrS}_2]/[\text{PrSH}] = K_2\,[\text{GSSG}]/[\text{GSH}]^2 \qquad \text{(Eq. 4)}$$

Therefore, the same change in the GSH/GSSG ratio will cause a significantly greater alteration in the PrSH/PrS_2 ratio compared with that in the PrSH/PrSSG ratio (39, 40, 94).

Thus, a regulatory switch depending on formation of a protein disulfide will be more sensitive to the GSH/GSSG ratio than one depending on formation of a mixed disulfide with GSH (38, 94). The formation of an internal disulfide is therefore more sensitive than the formation of a mixed disulfide to changes in the glutathione redox state, and may enable proteins containing vicinal thiols to respond to smaller changes in thiol redox state than those required to affect a lone protein thiol (41, 94). Consistent with this, vicinal dithiols are widespread *in vivo* (41). A final important consideration of the interaction between protein thiols and the GSH pool to note is that the extent of a reaction at a given GSH/GSSG ratio varies with [GSH], because the GSSG/2GSH reduction potential is dependent on $[GSH]^2$. This is shown in Eq. 5, which gives the $E_{h,8}$ value at 25°C: this was derived from $E^{\circ\prime}$ value of −240 mV at 25°C (40, 94): the $E_{h,8}$ values at 37°C are likely to be marginally higher.

$$E_{h,8}\,(\text{mV}) = -299.1 - 29.6 \log_{10}([GSH]^2/[GSSG]) \qquad \text{(Eq. 5)}$$

Therefore, the formation of mixed disulfides is only affected by the GSH/GSSG ratio, whereas the $PrSH/PrS_2$ ratio and the GSSG/2GSH reduction potential are affected by [GSH]. Although these differences are subtle, they may be important when the [GSH] changes, for example, during GSH efflux from apoptotic cells (111).

In summary, thiol protein function may be altered by glutathionylation either directly in response to the GSH/GSSG ratio or independently of this ratio by direct reaction of NO, H_2O_2, or peroxynitrite with protein thiols, followed by glutathionylation. These are appealing regulatory mechanisms; however, the proteins that are affected, the mechanisms, and the physiological significance are uncertain.

MITOCHONDRIAL PROTEINS AFFECTED BY GLUTATHIONYLATION

Exposure of mitochondria to oxidative stress oxidizes the mitochondrial glutathione pool, leading to extensive formation of persistently glutathionylated proteins and to protein disulfides (6, 89, 93). These changes are associated with disruptions to oxidative phosphorylation and with induction of the mitochondrial permeability transition (7, 27, 52, 64, 87, 110). Furthermore, alterations to the mitochondrial GSH/GSSG ratio are thought to be particularly important in mitochondria during apoptosis and necrosis, thereby contributing to a range of pathologies, including Parkinson's disease and Friedreich's ataxia (5, 7, 13, 19, 37, 93, 94). In support of this, changes in mitochondrial thiol redox state and the extrusion of GSH from the cell occur early in apoptosis and lead to mitochondrial changes and cytochrome *c* release (18, 33, 37, 45, 69, 95, 111). These and other findings suggest that mitochondrial thiol alterations may be part of the process by which the cell commits to apoptosis, as well as part of the response of mitochondria to oxidative damage.

In exploring all these processes, it is important to identify those proteins whose thiol redox state is affected by oxidative stress or redox signaling, determine whether the changes are due to the formation of an intraprotein disulfide or to persistent glutathionylation, find out which cysteine residues are affected, and explain how the redox changes affect protein function. In studies on mitochondrial membranes exposed to oxidized GSH/GSSG ratios, there was extensive loss of protein thiols that was largely due to the formation of intraprotein disulfides and only minimally due to proteins becoming persistently glutathionylated (6). Although techniques are being developed to analyze further the proteins affected by glutathionylation (10, 20, 31, 32, 42, 66), at present we know little about the details of these proteins and the physiological consequences of oxidation of their thiols.

Complex I is one of the mitochondrial proteins that has been shown to be persistently glutathionylated under conditions of oxidative stress (6, 108). Complex I is a large mitochondrial inner membrane protein of ~1 MDa that contains 46 polypeptide subunits, a flavin mononucleotide cofactor, and a number of iron-sulfur centers (48). Its principal role is as an NADH-ubiquinone oxidoreductase that is coupled to proton pumping across the mitochondrial inner membrane and that acts as a gateway for electrons into the respiratory chain (48). Glutathionylation does not lead to a direct alteration of the activity of complex I; instead, the duration of exposure to an oxidized GSH/GSSG ratio is critical for inactivation (6). These changes in complex I activity are of considerable pathological significance as the selective loss of complex I activity in the substantia nigra of brains from Parkinson's disease patients is associated with oxidation of the glutathione pool (22, 51). Complex I has a number of other important contributions to the mitochondrion: it is a major source of ROS within the cell (99); it is involved in the mitochondrial permeability transition (29); it is particularly susceptible to inactivation during degenerative diseases and other pathologies (16, 51); and it is also involved in early mitochondrial changes during apoptosis (90). The central role of complex I in mitochondria suggests that the Grx2-catalyzed glutathionylation of complex I in response to slight oxidation of the mitochondrial glutathione pool may have physiological significance, although what this might be is not clear at present.

A number of other mitochondrial proteins have been identified as being glutathionylated in mitochondria. Cytochrome oxidase subunit Va within human T lymphocyte cells was glutathionylated in response to diamide (31), and cytochrome oxidase subunit Vb in rat hepatocytes was glutathionylated following exposure to menadione (32). Again, the physiological significance of this alteration is unclear. α-Ketoglutarate dehydrogenase is reversibly inactivated under conditions that led to oxidation of the mitochondrial GSH pool and this was reversed by Grx, suggesting that its inactivation may be due to glutathionylation (78). There have also been reports of the glutathionylation of the E2 subunit of pyruvate dehydrogenase during apoptosis in HeLa cells (79) and of aconitase during heart ischemia–reperfusion injury (24). However, the identification of glutathionylated mitochondrial proteins and exploration of the significance of these modifications are still in their infancy.

CONCLUSIONS

The interaction of the mitochondrial glutathione pool with protein thiols is an important aspect of mitochondrial biology. There is rapid, extensive, and reversible interplay between the

redox state of mitochondrial membrane protein thiols and the glutathione pool. These interactions occur by thiol–disulfide exchange and are catalyzed by Grx2, enabling protein thiols to respond rapidly to a wide range of GSH/GSSG ratios during oxidative damage and redox signaling. In addition, protein thiols may also become glutathionylated independently of the redox state of the GSH pool, for example, following *S*-nitrosylation or sulfenic acid formation. Further investigation of the role of protein glutathionylation within mitochondria will improve our understanding of oxidative damage, cell death, and redox signaling.

ABBREVIATIONS

BSO, buthionine sulfoximine; Gpx, glutathione peroxidase; GR, glutathione reductase; Grx, glutaredoxin; GSH, glutathione; GSNO, *S*-nitrosylated glutathione; GSSG, glutathione disulfide; H_2O_2, hydrogen peroxide; NO, nitric oxide; $O_2^{\bullet -}$, superoxide; PlOOH, phospholipid hydroperoxides; PrSH, protein thiol; $Pr(SH)_2$, vicinal dithiol protein; PrS_2, intraprotein disulfide; PrSSG, protein-glutathione mixed disulfide; RNS, reactive nitrogen species; ROS, reactive oxygen species; Trx, thioredoxin; TrxR, thioredoxin reductase.

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