Disulphide formation on mitochondrial protein thiols

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Abstract
A large number of proteins contain free thiols that can be modified by the formation of internal disulphides or by mixed disulphides with low-molecular-mass thiols. The majority of these latter modifications result from the interaction of protein thiols with the endogenous glutathione pool. Protein glutathionylation and disulphide formation are of significance both for defence against oxidative damage and in redox signalling. As mitochondria are central to both oxidative damage and redox signalling within the cell, these modifications of mitochondrial proteins are of particular importance. In the present study, we review the mechanisms and physiological significance of these processes.

Introduction
There are a variety of mitochondrial PrSHs (protein thiols), which can be divided into (i) essential thiols in the active sites of enzymes, (ii) thiols exposed on the surface of proteins and (iii) buried thiols. It is the exposed thiols that are of interest here since these include regulatory PrSHs and thiols involved in antioxidant defence. Within mitochondria, these exposed thiols are present at a high concentration [1,2]. GSH is the predominant low-molecular-mass thiol within the cytosol and mitochondria [3–8]. GSH is an important protection against oxidative damage, both by direct reaction with ROS (reactive oxygen species) or reactive nitrogen species and as an electron donor for peroxidases [3,5]. GSH is oxidized to GSSG by ROS and by GPXs (glutathione peroxidases) [9–11]. GR (glutathione reductase) continually recycles GSSG back to GSH [3,7,12] keeping the GSH/GSSG ratio in mitochondria high; typically 95–99% is reduced, except during oxidative stress [3].

Protein glutathionylation
PrSHs can interact with mitochondrial glutathione by many mechanisms. A major pathway is thiol–disulphide exchange between a free PrSH and GSSG (Reaction 1):

\[ \text{PrS}^- + \text{GSSG} \rightarrow \text{PrS-SG} + \text{GS}^- \]

The high GSH/GSSG ratio minimizes protein glutathionylation [11]; however, during oxidative stress, PrSHs respond to the decreased GSH/GSSG ratio by forming mixed disulphides with glutathione. The reactivity of PrSHs with GSSG depends on the pH of the thiol, typically 8–9, but these can be altered dramatically depending on the local environment [13,14]. This variability in thiol reactivity may enable exposed thiols to respond by altering their glutathionylation status in a graded fashion to changes in the GSH/GSSG ratio.

Another cause of protein glutathionylation is oxidative damage to the PrSH. Oxidation of a thiol [15,16] can yield a thyl radical (RS•), which can then react with a glutathionylate anion (GS−) to form a radical mixed disulphide (RSSG•−), which will lose an electron to oxygen to form superoxide radical (O2•−), leaving a mixed disulphide [17] (Reaction 2):

Another route to mixed disulphides is through the two-electron oxidation of a thiol to a sulphenic acid (RSOH), which will then react with a thiolate anion to displace OH− (Reaction 3):

\[ \text{PrSOH} + \text{GS}^- \rightarrow \text{PrS-SG} + \text{OH}^- \]

Mitochondria are exposed to excess NO during pathological conditions [18,19] and this can lead to the formation of peroxynitrite (ONOO−), which can oxidize thiols to either thyl radicals or to sulphenic acids and lead to protein glutathionylation as above [18,20,21]. It is also possible that S-nitrosylation of a PrSH to form a PrSNO can lead to protein glutathionylation by the displacement of the nitroxyl anion (NO−) by glutathione [22–25] (Reaction 4):

\[ \text{PrSNO} + \text{GS}^- \rightarrow \text{PrS-SG} + \text{NO}^- \]

Fate of glutathionylated proteins
The above mechanisms can all form protein–glutathione mixed disulphides. Although the disulphide bond is readily reversible, the glutathionylation can be maintained indefinitely under oxidizing conditions [13,26]. However, in
many situations, glutathionylation is only transient since an adjacent PrSH displaces the GSH to form a PrS^2 (intraprotein disulphide) (Reaction 5) [13]:

\[
\begin{align*}
\text{Pr-SG} + \text{GSH} &\rightarrow \text{Pr-S} + \text{GS}^- \\
\text{Pr-S} + \text{GS}^- &\rightarrow \text{Pr-S} + \text{GSH}
\end{align*}
\]

Thus there are two classes of glutathionylated proteins, transient and persistent [26]. While a wide range of mitochondrial membrane proteins contain exposed, reactive thiols that react with GSSG, only a few thiol proteins remain glutathionylated with most of them forming PrS^2 (Reaction 5) [26]. The mechanistic reason for this is presumably that glutathionylation normally occurs on PrSHs that are adjacent to a second thiol that rapidly displaces GSH (Reaction 5). This juxtaposition could arise by chance; however, both redox regulation and antioxidant defence might favour the formation of PrS^2 over mixed disulphides.

Once the GSH/GSSG ratio has returned to its resting state, the high GSH/GSSG ratio will reverse glutathionylation by thiol–disulphide exchange (Reaction 6):

\[
\text{PrS-SG} + \text{GSH} \rightarrow \text{PrS} + \text{GSSG}
\]

If the protein has formed an internal disulphide, then thiol–disulphide exchange with GSH will lead to the reduction of the disulphide via transient glutathionylation (Reaction 7):

\[
\begin{align*}
\text{PrS-SG} + \text{GSH} &\rightarrow \text{PrS-SH} + \text{GSSG} \\
\text{PrS-SH} + \text{GSH} &\rightarrow \text{PrS-SG} + \text{GS}^-
\end{align*}
\]

The reversal of glutathionylation and the reduction of PrS^2 can also be catalysed by the enzymes Grx (glutaredoxin) and Trx (thioredoxin).

### Grx

For both regulatory and antioxidant roles, it is important for the PrSH redox state to respond rapidly to changes in the GSH/GSSG ratio. Thiol–disulphide exchange between GSSG and a PrSH (Reaction 1) and the reverse reaction between a glutathionylated protein and GSH (Reaction 6) are catalysed by the protein Grx [13–15,27–30]. Grx has a solvent-exposed cysteine residue that is necessary for glutathionylation/deglutathionylation, facilitated by the adjacent glutathione-binding site and by its very low pK_a [29,31]. The Grx–GSH mixed disulphide intermediate is reduced by GSH [29,31,32]. Recently, a mitochondrial isoform, Grx2, has been discovered [33–35] that catalyses both the glutathionylation and deglutathionylation of mitochondrial thiol proteins and the reduction of protein disulphides [26,35]. This supports a role for exposed PrSHs in mitochondrial antioxidant defence (Reactions 2 and 3). Interestingly, the oxidation and persistent glutathionylation of PrSHs by GSSG were catalysed dramatically by Grx2, even at relatively decreased GSH/GSSG ratios [26], indicating that Grx2 could contribute to regulatory changes in glutathionylation. Thus Grx2 stands at the centre of the reversible interactions of PrSHs with the mitochondrial glutathione pool (Scheme 1).

### Trx

Trx proteins are small thiol proteins that have an active-site dithiol that reduces protein disulphides, leaving an internal disulphide on Trx [37,38]. The disulphide form of Trx is reduced to its active dithiol by TrxR (Trx reductase) [37]. Mitochondria contain their own Trx (Trx2) [39,40] and TrxR (TrxR2) [41–44]. The major function of Trx2/TrxR2 in mitochondria is likely to be reduction of PrS^2 [37,38] (Reaction 8):

\[
\begin{align*}
\text{PrS-SH} + \text{Tr(SH)}_2^- &\rightarrow \text{PrS-S} + \text{Tr(S)}_2^- \\
\text{PrS-S} + \text{Tr(S)}_2^- &\rightarrow \text{PrS-SH} + \text{Tr(SH)}_2^-
\end{align*}
\]

### Physiological roles of mitochondrial protein glutathionylation

The reversible glutathionylation of PrSHs is thought to have two biological functions: antioxidant defence and redox signalling. As the concentration of exposed reactive PrSHs in mitochondria is greater than that of GSH, the direct reaction of PrSHs with ROS may be important for antioxidant defences [15,16]. Reactions with ROS convert PrSHs into thiol radicals or sulphenic acids, which can be further oxidized...
to higher oxidation states such as sulphinic (RSO₂H) and sulphonic (RSO₃H) acids [15,16]. The rapid reactions of protein thiyl radicals or sulphinic acids with GSH prevent the formation of these higher oxidation states. This reaction with GSH to form a radical mixed disulphide may be a major pathway for the repair of thiyl radicals in vivo [17]. The protein mixed disulphide can then be reduced back to a PrSH by GSH (Reaction 6).

Another putative antioxidant role for the formation of protein mixed disulphides is to buffer the GSH/GSSG ratio, by reacting with GSSG to release GSH, leaving a protein mixed disulphide (Reaction 1) or a protein disulphide (reverse of Reaction 7) [16]. This will minimize oxidation of the glutathione pool during transient oxidative stress [16]. Once the oxidative stress has subsided, the protein mixed disulphide or PrS₂ will be reduced back to a PrSH by GSH (Reactions 6 and 7), Trx2 [39] (Reaction 8) or Grx2 [33,34]. However, the significance of this process in vivo is uncertain.

In addition to antioxidant functions, there is growing evidence suggesting a role for protein glutathionylation in redox sensing and signalling [16,45–49]. Both glutathionylation and the formation of PrS₂ can dramatically affect the activity of enzymes, transcription factors and transporters, enabling them to respond reversibly to the ambient GSH/GSSG ratio [16,32,45–48,50]. Supporting such a role, many enzymes and proteins undergo alterations in activity on glutathionylation or on the formation of PrS₂ [16,45,46]. However, glutathionylation is less selective than residue-specific phosphorylation and dephosphorylation by kinases and phosphatases. Even so, the selectivity of a particular PrSH may be strongly influenced by the pKa and accessibility of the cysteine residue. In addition, PrSH glutathionylation may arise on particular PrSHs following their transformation into S-nitrosothiols (Reaction 4) or sulphinic acids (Reaction 3) formed from a reaction with ONOO⁻ or H₂O₂. Furthermore, glutathionylation and deglutathionylation can be catalysed by Grx, which may help to form or degrade mixed disulphides at particular PrSHs and thus modulate the lifetime of critical glutathione–protein mixed disulphides [15]. While these suggestions are speculative, a corollary is that protein glutathionylation may be both a general response to oxidative stress with bulk changes to most exposed PrSHs [16,27,46] and also a selective modulation of a small group of critical regulatory thiols. Furthermore, there does seem to be a level of basal protein glutathionylation in resting cells despite a fully reduced GSH pool [45,49].

Conclusions
The interaction of the mitochondrial glutathione pool with PrSHs is an important aspect of mitochondrial biology. There is rapid, extensive and reversible interplay between the redox state of mitochondrial membrane PrSHs and the glutathione pool. These interactions occur by thiol–disulphide exchange and are catalysed by Grx2, enabling PrSHs to respond rapidly to a wide range of GSH/GSSG ratios during oxidative damage and redox signalling. Future work on PrSH modifications within mitochondria will extend our understanding of a range of processes including oxidative damage and redox signalling.

References

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