Glutaredoxin 2 Catalyzes the Reversible Oxidation and Glutathionylation of Mitochondrial Membrane Thiol Proteins

IMPLICATIONS FOR MITOCHONDRIAL REDOX REGULATION AND ANTIOXIDANT DEFENSE‡

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The redox poise of the mitochondrial glutathione pool is central in the response of mitochondria to oxidative damage and redox signaling, but the mechanisms are uncertain. One possibility is that the oxidation of glutathione (GSH) to glutathione disulfide (GSSG) and the consequent change in the GSH/GSSG ratio causes protein thiols to change their redox state, enabling protein function to respond reversibly to redox signals and oxidative damage. However, little is known about the interplay between the mitochondrial glutathione pool and protein thiols. Therefore we investigated how physiological GSH/GSSG ratios affected the redox state of mitochondrial membrane protein thiols. Exposure to oxidized GSH/GSSG ratios led to the reversible oxidation of reactive protein thiols by thiol-disulfide exchange, the extent of which was dependent on the GSH/GSSG ratio. There was an initial rapid phase of protein thiol oxidation, followed by gradual oxidation over 30 min. A large number of mitochondrial proteins contain reactive thiols and most of these formed intraprotein disulfides upon oxidation by GSSG; however, a small number formed persistent mixed disulfides with glutathione. Both protein disulfide formation and glutathionylation were catalyzed by the mitochondrial thiol transferase glutaredoxin 2 (Grx2), as were protein deglutathionylation and the reduction of protein disulfides by GSH.

Complex I was the most prominent protein that was persistently glutathionylated by GSSG in the presence of Grx2. Maintenance of complex I with an oxidized GSH/GSSG ratio led to a dramatic loss of activity, suggesting that oxidation of the mitochondrial glutathione pool may contribute to the selective complex I inactivation seen in Parkinson’s disease. Most significantly, Grx2 catalyzed reversible protein glutathionylation/deglutathionylation over a wide range of GSH/GSSG ratios, from the reduced levels accessible under redox signaling to oxidized ratios only found under severe oxidative stress. Our findings indicate that Grx2 plays a central role in the response of mitochondria to both redox signals and oxidative stress by facilitating the interplay between the mitochondrial glutathione pool and protein thiols.

Oxidative damage and redox signaling can regulate protein thiol redox state (1–4). A major way in which this occurs is through the response of protein thiols to changes in the glutathione (GSH) to glutathione disulfide (GSSG) ratio (2, 5, 6). The intracellular GSH/GSSG ratio is usually kept high (>99% reduced) through reduction of GSSG to GSH by glutathione reductase, enabling GSH to act as an antioxidant (2, 6). However, during oxidative stress or redox signaling reactive oxygen species (ROS)1 oxidize GSH to GSSG directly, or catalyzed by glutathione peroxidases. Protein thiols respond to the decreased GSH/GSSG ratio by forming mixed disulfides with glutathione through thiol-disulfide exchange between the thiolate anion and GSSG (protein thiols typically have pKₐ values of ~8–9, but these can vary widely depending on the local environment of the cysteine residue, Ref. 1) (Reaction 1).

\[
\text{Pr}SH + \text{GSSG} \rightarrow \text{PrS-SG} + \text{GS}^- \\
\text{REACTION 1}
\]

The protein glutathione-mixed disulfide can be maintained as a persistently glutathionylated protein, or an adjacent protein thiol can displace the GSH to form an intraprotein disulfide as shown in Reaction 2 (1).

\[
\text{Pr-S-SG} \rightarrow \text{Pr-S} + \text{GS}^- \\
\text{REACTION 2}
\]

After the oxidative stress or redox signal has subsided, glutathione reductase will return the GSH/GSSG ratio to its resting level, enabling reversal of the protein thiol redox changes (see Reactions 3 and 4), although protein disulfides can be reduced by thioredoxin 2 (Trx2) (7, 8) and glutaredoxin 2 (Grx2) (9, 10).

1 The abbreviations used are: ROS, reactive oxygen species; BCA, bicinchoninic acid; DDM, dodecyl-β-d-maltoside; DTPA, N,N-bis(2-bis(carboxymethyl) aminoethyl) glycine; DTNB, dithionitrobenzoic acid; DTT, dithiothreitol; sGSH, antiserum against glutathionylated protein; Grx, glutaredoxin; HEDS, β-hydroxyethylene disulfide; IAM, iodoacetamide-biotin; IBTP, (4-iodo)butyltriphenylphosphonium iodide; MAL, maleimide-biotin; MBTA, 4-(N-maleimidobenzyl)-α-trimethlylammonium iodide; MTSET, [2-trimethylammonium(ethyl)methanethiosulfonate bromide; NEM, N-ethylmaleimide; PrSH, protein thiol; PrS·, intraprotein disulfide; Trx, thioredoxin; BSA, bovine serum albumin; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; PVDF, polyvinylidene difluoride; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; BN-PAGE, Blue Native-PAGE.
Alteration to protein thiol redox state via changes in the GSH/GSSG ratio during oxidative stress and redox signaling is thought to be particularly important in mitochondria during apoptosis, necrosis, and induction of the permeability transition, although the details are uncertain (2, 11–16). The mitochondrial glutathione pool is separate from that of the cytosol (17) with mitochondria having their own glutathione reductase, glutathione peroxidases, and NADPH sources (16, 18, 19). Consequently the mitochondrial and cytosolic GSH/GSSG ratios can vary independently (17).

The concentration of reactive protein thiols in cells is greater than that of GSH, and the interaction of protein thiols with the glutathione pool is important for antioxidant defense (2, 20, 21). Reactions with ROS convert protein thiols to thyl radicals or sulfenic acids, which can be further oxidized (2, 20, 21). To prevent this irreversible protein oxidation, GSH reacts with protein sulfenic acids (Reaction 5) and thyl radicals (Reaction 6) to form mixed disulfides (2, 20–22). The protein-mixed disulfide can then be reduced back to a protein thiol by GSH (Reaction 4).

Some exposed, non-catalytic protein thiols may also help 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 (Reaction 2) (21). This will maintain the glutathione pool in a reduced state during transient oxidative stress (2, 21). Once the oxidative stress has subsided, the protein-mixed disulfide or intraprotein disulfide will be reduced back to a protein thiol by GSH (Reactions 3 and 4), Trx2 (8), or Grx2 (9, 10).

Reaction of the mitochondrial GSH/GSSG ratio with protein thiols is also involved in the regulation of mitochondrial function in response to redox signaling and oxidative stress (2, 16). Both glutathionylation and the formation of intraprotein disulfides can dramatically affect the activity of enzymes and transcription factors, enabling them to respond reversibly to the ambient GSH/GSSG ratio, just as proteins are regulated by reversible phosphorylation (2–5, 23–25). Although directly altering thiol protein function in response to the GSH/GSSG ratio is an appealing regulatory mechanism, the proteins affected, the mechanisms, and the physiological significance are uncertain.

For both regulatory and antioxidant roles it is important for 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) or between a glutathionylated protein and GSH (Reaction 4) is often relatively slow (1, 26, 27). A potential catalyst for thiol-disulfide exchange is the small, soluble protein glutaredoxin (Grx) (28). Grx from *Escherichia coli* has a CPYC active site motif with a solvent-exposed Cys-11, while 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 (29). Only Cys-11 is necessary for glutathionylation/deglutathionylation, facilitated by the adjacent glutathione binding site and by its low pK (30). Grx catalyzes the deglutathionylation of protein-glutathione mixed disulfides (Reaction 4) far more effectively than thioredoxin (Trx) or protein-disulfide isomerase (20). Recently a mitochondrial isoform, Grx2, has been discovered which has a N-terminal mitochondrial targeting peptide that yields a mature protein of about 15 kDa (9, 10). 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 just like Cys-11 in *E. coli* (31). Modeling suggests that the GSH binding site and the hydrophobic surface of Grx2 are similar to those of Grx1 (9, 10). However, there are significant differences between the two isoforms: Grx2 lacks one of the conserved non-active site Cys residues of Grx1 (10) and is consequently less easily inactivated by oxidants and GSSG (9); in addition, Grx2 can be reactivated directly by thioredoxin reductase as well as by GSH (31). These differences may help Grx2 to operate in the more oxidatively stressed mitochondrial environment. Therefore Grx2 is a candidate for catalyzing the interplay of the mitochondrial glutathione pool with protein thiols during both antioxidant defense and redox signaling.

Here we have investigated the interaction of the GSH/GSSG ratio with mitochondrial thiol proteins. In particular we were curious to know whether protein thiol redox changes occurred under physiologically accessible GSH/GSSG ratios and whether these were catalyzed by Grx2. In addition, we wished to determine whether the reaction of mitochondrial protein thiols with GSSG led to the formation of persistently glutathionylated proteins, or to intraprotein disulfides, and how these processes depended on the GSH/GSSG ratio. To answer these questions we incubated mitochondrial membranes with a range of GSH/GSSG ratios and with Grx2. The extent of oxidation of mitochondrial membrane protein thiols by GSSG through thiol/disulfide exchange was dependent on the GSH/GSSG ratio and was catalyzed by Grx2. Most protein thiols formed intraprotein-mixed disulfides with only a few, notably complex I, forming persistent mixed disulfides with glutathione. Importantly, these protein thiol redox changes were sensitive to mild oxidation of the glutathione pool. These findings suggest that Grx2 enables mitochondrial protein thiols to respond rapidly and reversibly to a wide range of GSH/GSSG ratios, contributing to both redox regulation and antioxidant defenses within mitochondria.

**EXPERIMENTAL PROCEDURES**

**Materials**—(4-Iodo)butyltriphenylphosphonium iodide (IBTP) and 3-trifluoromethylaryltrifluoroacetone (THTA) rabbit antiserum were prepared as described (32). Rabbit antiserum against the 75, 51, and 23 kDa bovine complex I subunits were from Dr. John E. Walker. Maleimide-biotin (MAL) and iodoacetamide-biotin (IAM) were from Pierce Biotechnology and were detected using extravidin-horseradish peroxidase from Sigma. Anti-GSH IgG2a mouse monoclonal was from Virogen and was detected using an anti-mouse IgG-horseradish peroxidase conjugate from Sigma. Anti-GSH IgG2a mouse monoclonal was from Virogen and was detected using an anti-mouse IgG-horseradish peroxidase conjugate from Sigma. To confirm the selectivity of this antibody for glutathionylated proteins, we used BSA that had been reduced by mercaptoethanol, dialyzed and then glutathionylated by incubation with GSSG. The antibody detected a single band of ~68 kDa on immunoblots following non-reducing SDS-PAGE, and antibody binding was prevented by treating the glutathionylated BSA with dithiothreitol (DTT) or by preincubation of the antibody with 5 mM GSSG. Complete protease inhibitor was from Roche Applied Science. Cellulose-coated HPTLC plates (10 cm²) were from Merck. [35S]GSH (750–950 Ci/mol) was from PerkinElmer Life Sciences. To prevent the DTT (10 mM) present in the [35S]GSH stock solution from interfering with experiments using low (<200 μM) glutathione concentrations, the DTT was removed by dilution of an aliquot of the [35S]GSH stock to ~50–70 μl in KP buffer (50 mM KP, 1 mM EGTA, 100 μM DTPA, pH 8) and extracting this with 4 × 1 ml CHCl₃. Ferrocytochrome c was prepared by reduction of bovine heart cytochrome c (Sigma) with...
Glutaredoxin 2 and Mitochondrial Protein Thiols

Preparation and Incubation of Mitochondria, Membranes, and Complex I—Bovine heart mitochondria were prepared as described (32). Bovine heart mitochondrial membranes were prepared by disruption of mitochondria in a blender, followed by collection and washing by centrifugation (34). These mitochondrial membrane preparations had negligible matrix contamination, as indicated by the lack of MnSOD detected by immunoblotting and a citrate synthase specific activity in membranes that was <10% relative to intact bovine heart mitochondria. These preparations were simply open fragments of mitochondrial membranes and did not contain closed vesicles. This is illustrated by the rapid respiration of the membranes on both NADH and ferrocytochrome c, which are membrane impermeant electron donors that pass electrons to the respiratory chain from opposite sides of the mitochondrial inner membrane. Furthermore, the rate of ferrocytochrome c oxidation was not increased on addition of the detergent Triton X-100 over permeability. To measure exposure of thiols by the blocked NADH-linked respiration, indicating that destroying membrane integrity did not increase accessibility of ferrocytochrome c to cytochrome c oxidase. For most experiments bovine heart mitochondrial membranes were preincubated at 1 mg of protein/ml with 1 mM DTT in KP buffer for 10 min at 37 °C. The membranes were then pelleted by centrifugation (15,000 × g) and washed in KP buffer. Complex I was separated by gel electrophoresis with densitometer (9). Linear (0–0.5% w/v) ammonium sulfate precipitation, and gel filtration, and the pure complex I was stored in buffer further purified by ion-exchange separation, ammonium sulfate precipitation, and gel filtration, and the complex pure complex I stored in buffer

In assay, duplicate membrane samples were pelleted by centrifugation (37.5 mTris-HCl, 7. pH, 3. Sodium borohydride (1%/v final) was then added, the samples vortexed, and incubated at 40 °C for 30 min. The proteins were precipitated with 10% sulfosalicylic acid (15 min at room temperature, then 15 min on ice, followed by centrifugation at 15,000 × g for 15 min). The GSH released into the supernatant was assayed by the recycling assay and compared with GSH standards (0–0.2 nmol). The recycling assay was done using a 96-well plate and consisted of 13.5 μl of sample or standard, mixed with 285 μl of 0.5 mM DTNB, 0.5 mM NADPH in 85 mM NaPi 3.7 mM EDTA, pH 7.5, and 1 unit of glutathione reductase (Sigma) (11). The formation of thionitrobenzoic acid was measured at 405 nm over 10 min using a kinetic plate reader (EL, 808 ultramicroplate reader, Bio-Tek Instruments Inc.).

Expression, Isolation, and Characterization of Glutaredoxin 2—A pET21d (+)-Grx2 plasmid encoding the His-tagged version of the mature mouse Grx2 protein was kindly provided by Dr. Vadim N. Chernikov. Bovine heart mitochondria were prepared as described (33).

342°C the lysate was centrifuged at 12,000 × g for 10 min at 4 °C, and the supernatant was centrifuged again at 160,000 × g for 3 h at 4 °C. The supernatant from the second centrifugation was applied to a GSH-Sepharose gel equilibrated with 0.5 mM NaCl, 20 mM Tris-HCl, pH 7.9, using a Dounce homogenizer and lysed with a French Pressure cell (Sim-Amino Spectronic Instruments). The crude lysate was centrifuged at 12,000 × g for 10 min at 4 °C, and the supernatant was centrifuged again at 160,000 × g for 3 h at 4 °C. The supernatant from the second centrifugation was applied to GSH-Sepharose gel equilibrated with 0.5 mM NaCl, 20 mM Tris-HCl, pH 7.9. The protein was then eluted using an imidazole gradient from 0 to 1 M over 100 min. Grx2 eluted at ~0.45 M. Pooled fractions were dialyzed overnight against 1 mM KP, pH 7.5 with three changes of medium, with 2 mM DTT in the first dialysis. The protein gave a single band of the expected size by SDS-PAGE and Tris-Tricine gels. The protein concentration was determined using the BCA assay with BSA as a standard (37), and the protein was stored at ~80 °C in 1 mM KP, pH 7.5 for 2.5–5 mg protein/ml. Grx2 enzyme activity was measured in 1 ml of 0.1 M KP, pH 7.4 containing 0.2 mM NADPH, 0.7 mM β-hydroxyethylene disulfide (HEDS), 0.5 mM GSH and 0.4 units/ml glutathione reductase (9). After preincubation at 30 °C for 10 min, the background was recorded for 2 min, then 0–10 μg Grx2 was added, and the loss of NADPH followed for 30 min. The slope of the linear portion of the time course was corrected for background and the activity calculated (ε412 = 6,220 M −1 cm−1). Grx2 gave a specific activity of 7–9 units/mg protein consistent with published values (9, 10) (1 unit = 1 μmol NADPH/min). The activity of freshly isolated Grx2 was unaffected by a single cycle of freeze-thawing, so for experiments aliquots that had been stored at 80 °C were used with no additional treatment.

Electrophoresis and Immunoblotting—For SDS-PAGE samples were generally pelleted by centrifugation (membranes) or precipitated with acetone (complex I). For SDS-PAGE run under non-reducing conditions, samples were generally treated with 10% 5-sulfosalicylic acid on ice for 15 min, then pelleted by centrifugation, and resuspended in loading buffer lacking a thiol reductant but supplemented with 50 mM NEM.
Gels (usually 12.5% acrylamide or 12–22% acrylamide linear gradient gels) were run using a Bio-Rad Mini Protein system and transferred to PVDF overnight at 4 °C using a Bio-Rad Mini Protein Transfer Cell. The blot was incubated with anti-serum followed by an anti-body-horseradish peroxidase conjugate and visualized by enhanced chemiluminescence (ECL; Amersham Biosciences). Tris-Tricine gels were prepared and run as described (38).

For Blue Native (BN)-PAGE membranes were mixed with NEM (50 mM) after incubation, pelleted and 80–120 μg of protein was resuspended in 100 μl of extraction buffer (0.75 m aminoacetic acid, 50 mM Bis-Tris pH 7 at 4 °C) containing 1% DDM on ice for 15 min, then centrifuged (70,000 rpm for 15 min in a Beckman Airfuge; 60,000 × g). The supernatant was mixed with 5% Serva Blue in 500 mM aminoacetic acid, loaded on a 5–12 or 5–15% gradient gel in a Bio-Rad Mini Protein system and run overnight at 4 °C with ferritin as a molecular weight marker (39). For fluorography of 35S-labeled proteins, the BN-PAGE gel was fixed and stained in Coomassie Blue, soaked in Amplify (Amersham Biosciences), and dried and exposed to Fuji Medical x-ray film at −80 °C. For immunoblots following BN-PAGE, proteins were transferred to PVDF using the Bio-Rad Trans-Blot Semi-Dry transfer cell.

To identify proteins by peptide mass fingerprinting, bands were excised from Coomassie-stained SDS-PAGE gels and subjected to in-gel proteolysis with trypsin (40). The mixture of tryptic peptides was analyzed by MALDI-TOF MS using a ToFSpec 2E mass spectrometer (Micromass, Altrincham, UK) with α-cyano-4-hydroxy-trans-cinnamic acid as the matrix. Trypsin peptides at 2163.057 and 2273.160 and a matrix-related ion at 1060.048 were used to calibrate the spectra. The Mascot program (www.matrixscience.com) was used for data base searches.

Assays—Enzyme assays were performed at 30 °C. Complex I in mitochondrial membranes (90 μg protein/ml) was measured as the rotenone-sensitive oxidation of NADH (ε260 = 6,220 molar−1 cm−1) in KP, buffer supplemented with 2 mM KCN, 300 nM antimycin, 100 μM NADH, and 50 μM ubiquinone-1. The rotenone-sensitive rate was typically ~90% of the uninhibited rate. The complex II/III activity of bovine heart mitochondrial membranes (45 μg protein/ml) was measured in KP, buffer supplemented with 20 mM succinate, 2 mM KCN, and 4 μg/ml rotenone. After 5 min of pre-equilibration, 30 μM ferrocytochrome c was added, and the rate of reduction was measured (ε420 = 52,000 molar−1 cm−1). The antimycin A-insensitive rate was negligible. The complex IV activity of bovine heart mitochondrial membranes (2.5 μg protein/ml) was measured as the KCN-sensitive rate of ferrocytochrome c oxidation (ε420 = 6,28 molar−1 cm−1) in 200 mM Tris, 10 μM EDTA, pH 8.0, 100 μM ferrocytochrome c, 300 nM antimycin A, 4 μg/ml rotenone, and 0.3% Tween-80 (Ref. 41). The KCN-insensitive rate was negligible. Respiration rate of additions, 100 μM NEM, 250 μM MTSET, or 250 μM MBTA for 30 min, and the exposed thiols quantitated by the DTNB assay. Pretreatment with 10 mM GSH for 5 min at 37 °C also gave ~35 nmol of thiol/mg of protein. The open bar shows free thiols in membranes that had not been pretreated with DTT. Data are means ± range of duplicate incubations for a typical experiment of four. B, loss of membrane protein thiols on incubation with GSSG assessed by the DTNB assay. Mitochondrial membranes were incubated as in A for 1 min (filled bars) or 30 min (open bars), with no additions, 5 mM GSH/2.5 mM GSSG, or 5 mM GSSG. Data are means ± range of duplicate incubations for a typical experiment of three. C, loss of membrane protein thiols on incubation with GSSG assessed by the papain assay. Mitochondrial membranes were incubated as in A with no additions, with 10 mM GSH, with 5 mM GSH/2.5 mM GSSG, or with 5 mM GSSG. Data are a percentage of a control incubation at 1 min and are means ± S.D. of three experiments. D, redox equilibration of [35S]GSH with GSH and GSSG. (35S]GH (5 μCi/ml) was added to 10 mM GSH, 5 mM GSH/2.5 mM GSSG, or 5 mM GSSG in KP buffer and incubated at 30 °C under argon and incubated. Then 1-μl samples were spotted onto a HPTLC plate and developed in butanol:acetic acid:water (3:2:2) for 1.5 h, dried, and the radioactivity visualized using a Packard Cyclone phosphorimager. The RI values for GSH and GSSG under these conditions were 0.64 and 0.42, respectively, in agreement with the radiolabeled bands. E, quantitation of [35S]GSH bound to mitochondrial membranes. KP, buffer supplemented with 10 mM GSH/1 mM NADPH/0.4 units/ml glutathione reductase, 5 mM GSH/2.5 mM GSSG, or 5 mM GSSG was preincubated with 20 μCi/ml [35S]GSH for 30 min under argon. Then mitochondrial membranes (1 mg protein/ml) were added and incubated at 37 °C under argon for 1 min or 30 min, and membrane-bound [35S]GSH was quantitated by liquid scintillation counting. Binding of [35S]GSH to membranes was through a disulfide bond as DTT treatment of the membranes after incubation led to no detectable membrane-bound [35S]GSH. Data are means ± ranges for two independent experiments.
membranes was measured using a Clark type oxygen electrode (Rank Brothers, Bottisham, Cambridge, UK). For this, membranes (1 mg of protein/ml) were suspended in KPi buffer at 30 °C generally using 5 mM NADH or 100 μM ferrocytochrome c as respiratory substrates.

RESULTS

Quantitation of Reactive Protein Thiols on Mitochondrial Membrane Proteins—To investigate the interaction of mitochondrial protein thiols with glutathione, we used bovine heart mitochondrial membranes. These were chosen because they contain large amounts of the oxidative phosphorylation complexes and metabolite transporters that are critical for mitochondrial function. The total number of protein thiols present in mitochondrial membranes was 87 ± 5 nmol of thiol/mg of protein (mean ± range, n = 2), measured by dissolving DTT-treated membranes in SDS. However, this measurement includes structural thiols such as those forming iron-sulfur centers. As only protein thiols exposed on the surface of native proteins will respond to the GSH/GSSG ratio in vivo, we quantitated exposed protein thiols with DTNB without disrupting the membranes with detergent. The protein thiol content was the same ± Grx2 after 15 min of incubation (data not shown). B, stimulation of [35S]GSH binding to mitochondrial membranes by Grx2. DTT-treated mitochondrial membranes were incubated as described in the legend to Fig. 1E with 5 mM GSSG ± Grx2, or 10 mM GSH ± Grx2, and the amount of membrane-bound [35S]GSH was determined. Data are means ± S.E. of four independent experiments. The acceleration of glutathione mixed disulfide formation by Grx2 was also confirmed independently by the recycling assay (data not shown). C, effect of Grx2 on long term incubations with GSSG. Mitochondrial membranes were incubated as in Fig. 1E with 5 mM GSSG ± Grx2, and the amount of membrane-bound [35S]GSH was determined. Data are means ± range for duplicate determinations and show a typical experiment of two. D, reversibility of thiol loss by GSH and Grx2. Mitochondrial membranes were incubated as in Fig. 1A with 5 mM GSSG for 30 min then isolated, washed, incubated with no additions, or with 10 mM GSH ± Grx2, and the exposed thiols quantitated by the DTNB assay. Data are means ± range for two independent experiments. E, reversibility of thiol loss by GSH and Grx2 measured by the papain assay. Mitochondrial membranes were incubated as described in the legend to Fig. 1A with 5 mM GSSG and Grx2 for 10 min, then isolated, washed, and suspended at 1 mg protein/ml as in Fig. 1A for 2 min in duplicate with no further additions, 10 mM GSH/1 mM NADPH/0.4 units/ml GR, 10 mM GSH + Grx2, or 1 mM DTT. The protein thiols were then assessed in duplicate samples using the papain assay. Data are a percentage of total thiols recoverable on DTNB treatment and are means ± S.E. of seven experiments. *, p < 0.05 by Student’s t test for paired data. F, reversal of [35S]GSH binding to membranes by Grx2 and GSH. KPi buffer containing 5 mM GSSG was preincubated with 38 μCi/ml [35S]GSH for 30 min then mitochondrial membranes (4 mg protein/ml) were added and incubated for 30 min. The glutathionylated membranes were then pelleted, resuspended, and incubated as described in the legend to Fig. 1A with no additions, with 500 μM GSH ± Grx2, or Grx2 alone for 1 min. and the amount of membrane-bound [35S]GSH determined. Data are means ± range for duplicate assays and show a typical experiment of three.
nmol of thiol/mg protein exposed in native membranes, which increased to −35 nmol thiol/mg protein on pretreatment with the thiol reductants DTT or GSH, because of reduction of accumulated disulfides and sulfenic acids (Fig. 1A). All these protein thiols were accessible in native membranes, as the membrane-impermeable thiol alkylating reagents MTSET and MBTA blocked protein thiols to the same extent as the membrane-permeant NEM (Fig. 1A). Therefore the DTT-treated mitochondrial membranes that were used in subsequent experiments contain about 35 nmol/mg protein-exposed thiols that can potentially react with the glutathione pool.

**Incubation of Mitochondrial Membranes with GSSG Oxidizes Free Protein Thiols**—We next investigated how exposed mitochondrial protein thiols interacted with different GSH/GSSG ratios. These incubations were at pH 8 to mimic the alkaline mitochondrial matrix: as thiol-disulfide exchange occurs via the protein thiolate anion (pKa typically 8.5–9) thiol reactivity is particularly sensitive to pH in the physiological range. For most experiments the concentration of glutathione equivalents ([GSH] + 2[GSSG]) was 10 mM, the concentration within mitochondria in vivo (1, 19), while varying the GSH/GSSG ratio. It is important to maintain a physiological [GSH] as the extent of a reaction at a given GSH/GSSG ratio varies with [GSH]. This is because the GSSG/GSH reduction potential is dependent on [GSH] as is shown in Equation 1, which gives the Econd value at 25 °C. This value was derived from

$$E_{cond}(mV) = -299.1 - 29.6 \log_{10}(\text{[GSH]/[GSSG]})$$

(Eq. 1)

Incubating membranes with 5 mM GSSG, the maximum concentration in vivo, led to the loss of −25 nmol of the exposed thiols over 30 min (Fig. 1B). Incubation with 5 mM GSH/2.5 mM GSSG also led to significant but less extensive thiol loss over this time (Fig. 1B). Assaying the oxidation of exposed thiols by papain activation, an alternative assay that can also be applied to intact membranes, showed similar loss of thiols on incubation with 5 mM GSH/2.5 mM GSSG or 5 mM GSSG, and minimal loss on incubation with 10 mM GSH (Fig. 1C). Therefore exposure to an oxidized GSH/GSSG ratio leads to a rapid initial loss of protein thiols within a minute or so, the extent of which is dependent on the GSH/GSSG ratio. This is followed by a further gradual thiol loss; however even after 30 min of incubation with 5 mM GSSG, only about 60–70% of the exposed thiols were oxidized. All these incubations were carried out in argon-sparged buffer with iron chelators, which prevented the loss of protein thiols by direct oxidation (Fig. 1B). Therefore the oxidized glutathione pool reacts with the protein thiols by thiol-disulfide exchange with GSSG (Reaction 1) and not by forming protein thiol radicals or sulfenic acids (Reactions 5 and 6).

**Incubating Mitochondrial Membranes with GSSG Leads to Limited Persistent Glutathionylation**—The loss of exposed protein thiols in Fig. 1, B and C is by thiol-disulfide exchange with GSSG that initially forms a mixed disulfide between GSH and the protein thiol (Reaction 1). This mixed disulfide could either persist, or rearrange to form an intraprotein disulfide (Reaction 2). To distinguish between these we measured the number of glutathione residues bound persistently to mitochondrial membranes. To do this [35S]GSH was mixed with various GSH/GSSG ratios and allowed to equilibrate over 30 min through thiol/disulfide exchange, as was confirmed by HPTLC (Fig. 1D). Mitochondrial membranes were then incubated with various [35S]-equilibrated GSH/GSSG ratios for 1 min or 30 min and the amount of membrane-bound [35S]GSH quantitated by scintillation counting (Fig. 1E). The amount of membrane-bound GSSG increased with time and with oxidation of the GSH/GSSG ratio, and the maximum amount bound was −6–8 nmol GSH/mg protein (Fig. 1E). Displacing protein-bound GSH with borohydride, followed by measurement of released GSH by the recycling assay, gave similar results (data not shown). Comparison of Fig. 1, B and D shows that while 5 mM GSSG oxidizes −25 nmol of thiol/mg of protein of the 35 nmol of thiol present, only −7 nmol of glutathione/mg of protein bound to the membranes. Therefore on incubation with 5 mM GSSG −50% exposed protein thiols are oxidized to protein disulfides, −20% form persistent mixed disulfides with GSH, and −30% are not oxidized.

**Grx2 Catalyzes Protein Thiol Oxidation and Glutathionylation**—To determine whether protein thiol oxidation and glutathionylation by GSSG were catalyzed by Grx2 we incubated mitochondrial membranes with Grx2 and various GSH/GSSG ratios. Grx2 increased the rate of oxidation of mitochondrial membrane protein thiols by 5 mM GSSG over short time periods (Fig. 2A), but after 5 min (Fig. 2A) or 15 min (data not shown) the extent of thiol oxidation was similar ± Grx2. Protein thiol glutathionylation was also increased by Grx2 after a 5-min incubation (Fig. 2B), but when the incubation was extended to 30 min the amount of glutathione bound was the same ± Grx2 (Fig. 2C). Therefore Grx2 catalyzes both protein oxidation and glutathionylation by thiol-disulfide exchange with GSSG.

**Grx2 Catalyzes Membrane Protein Thiol Reduction and De-glutathionylation by GSH**—We next determined whether Grx2...
catalyzed the reduction by GSH of mitochondrial protein thiols that had been oxidized by GSSG. Membranes were incubated with GSSG to generate stably oxidized protein thiols (Fig. 2D). Incubation with GSH led to the recovery of most of the thiols, but GSH and Grx2 together were required for complete thiol recovery over 30 min (Fig. 2D). Incubation of GSSG-oxidized membranes for 2 min with GSH + Grx2 confirmed that Grx2 led to a statistically significant increase in the rate of reduction of GSSG-oxidized protein thiols by GSH (Fig. 2E). To see whether deglutathionylation was catalyzed by Grx2, glutathionylated mitochondrial membranes were prepared by incubation with GSSG. The protein-glutathione mixed disulfides were stable with GSH or with Grx2 alone, but Grx2 and GSH together removed about 50% of the protein-glutathione mixed disulfides after 1 min (Fig. 2F). Therefore Grx2 catalyzes both the deglutathionylation and the reduction by GSH of membrane protein thiols that have been oxidized by GSSG.

**Qualitative Analysis of the Protein Thiols Oxidized by GSSG and Grx2**—To complement the quantitation of thiol oxidation by GSSG, we visualized membrane proteins whose thiols had been altered by GSSG. This was done by labeling protein thiols with tags that can be detected on immunoblots (Fig. 3A). MAL reacted rapidly with all protein thiols; in contrast, both IAM and IBTP reacted slowly, labeling reactive thiols preferentially (32). Preincubating the membranes with GSSG/Grx2 completely blocked IBTP and IAM binding, but only slightly de-
increased MAL labeling, indicating that GSSG only modified reactive protein thiols (Fig. 3A). To confirm the thiol selectivity of IBTP and IAM, we measured their effects on exposed thiol content by the DTNB assay. MAL led to the complete loss of reactive protein thiols labeled by IBTP or IAM (Fig. 3A), indicating that reactive thiols are present on many mitochondrial membrane proteins.

Visualization of Persistently Glutathionylated Proteins—A proportion of membrane protein thiols should be stably glutathionylated following incubation with GSSG (Figs. 1 and 2). To visualize glutathionylated proteins we incubated membranes with GSSG and probed an immunoblot for glutathionylated proteins using an anti-GSH antibody (Fig. 4A). The samples were treated with NEM at the end of the incubation and dissolved in non-reducing loading buffer supplemented with NEM; these procedures stabilize protein-glutathione mixed disulfides and prevent the artifactual transfer of a GSH from one protein thiol to another during sample preparation. Only a few proteins of around 75 and 50 kDa in size were stably glutathionylated, and this was reversed by DTT (Fig. 4A). The number of stably glutathionylated proteins is far lower than that of reactive protein thiols labeled by IBTP or IAM (Fig. 3A), in agreement with the measurements in Fig. 1.

**Grx2 Catalyzes Protein Glutathionylation at Relatively Reduced GSH/GSSG Ratios**—The ready visualization of glutathionylated proteins on immunoblots enabled further investigation of the catalysis of protein glutathionylation by Grx2 (Fig. 4B). Incubating membranes with 5 mM GSSG for 5 and 15 s showed that protein glutathionylation was accelerated by Grx2 (Fig. 4B). Incubating membranes with 5 mM GSH/2.5 mM GSSG showed even greater acceleration of glutathionylation by Grx2 (Fig. 4C). The increased acceleration of glutathionylation by Grx2 at the more reduced GSH/GSSG ratio prompted us to measure the dependence of Grx2 catalysis on the GSH/GSSG ratio (Fig. 4D). This showed that while the glutathionylation of protein thiols over 1 min was slow at relatively reduced GSH/GSSG ratios, in the presence of Grx2 there was extensive protein glutathionylation even at relatively reduced GSH/GSSG ratios.

Reversal of Mitochondrial Protein Glutathionylation by Grx2—To investigate further protein deglutathionylation by
Grx2 we glutathionylated protein by incubation with GSSG (Fig. 4E). The protein glutathione mixed disulfides were stable when incubated with no additions, with Grx2 or with 500 μM GSH (Fig. 4E). However, incubation with GSH and Grx2 together led to rapid deglutathionylation within 15 s (Fig. 4E).

This confirms that Grx2 catalyzes the deglutathionylation of mitochondrial membrane proteins by GSH. We next compared the catalysis of protein glutathionylation by Grx2 with that of the ubiquitous dithiol protein Trx (Fig. 4F). Incubation of membranes with Grx2 and 5 mM GSH/2.5 mM GSSG led to rapid protein glutathionylation, but replacing Grx2 with Trx did not (Fig. 4F). Therefore the catalysis of glutathionylation is specific to Grx2 and is not a general property of dithiol proteins.

**Complex I Is a Major Glutathionylated Protein in Mitochondrial Membranes**—Proteins around 50 and 75 kDa were stably glutathionylated by GSSG. As the 75 kDa and 51 kDa subunits of complex I have reactive thiols and can be stably glutathionylated (42), they are likely candidates. To see if this was the case, we incubated membranes with GSSG, separated the proteins by non-reducing SDS-PAGE, and probed immunoblots for glutathionylated proteins and for the 75 and 51 kDa complex I subunits (Fig. 5A). This showed that the two glutathionylated protein bands at 50 and 75 kDa co-migrated with the complex I 51 and 75 kDa subunits, respectively (Fig. 5A). To extend this analysis we prepared a tryptic digest of the glutathionylated band at 75 kDa (Fig. 5B). Peptide mass finger printing by MALDI-ToF confirmed that this band contained the bovine complex I 75 kDa subunit (Fig. 5C). It was not possible to extend this analysis to the 50 kDa band due to the presence of multiple proteins in this region. This overlap was exacerbated by the diffuse bands found in non-reducing gels, furthermore the requirement for non-reducing conditions precluded the use of conventional two-dimensional gels. Finally, we confirmed that isolated complex I was stably glutathionylated on the 75 and 51 kDa subunits of complex I (Fig. 6A).

**Grx2 Catalyzes Glutathionylation of Complex I**—Grx2 dramatically catalyzed the glutathionylation and deglutathionylation of two proteins in mitochondrial membranes (Fig. 4), corresponding to the complex I 75 and 51 kDa subunits (Fig. 5). To confirm that Grx2 catalyzed complex I glutathionylation we incubated isolated complex I with a range of concentrations of GSSG ± Grx2 (Fig. 6A). At low GSSG concentrations (50–500 μM) there was negligible glutathionylation of complex I by

![Graph showing glutathionylation of complex I.](image)
GSSG alone over 1 min, however in the presence of Grx2 there was
extensive glutathionylation of the 75 kDa and 51 kDa
subunits (Fig. 6A). When isolated complex I was incubated with
different GSH/GSSG ratios there was also rapid glutathiony-
lation even at quite reduced GSH/GSSG ratios (Fig. 6B). At the
more oxidized GSH/GSSG ratios Grx2 catalysis was less evi-
dent, presumably due to rapid spontaneous glutathionylation
under these conditions.

To demonstrate further that Grx2 catalyzed the glutathioni-
ylation of complex I, we incubated mitochondrial membranes with
[35S]GSSG and assessed complex I glutathionylation by fluoro-
graphy of BN-PAGE gels (Fig. 7A). Long-term incubation of mito-
ochondrial membranes with GSSG led to complex I glutathiony-
lation that was reversed by DTT (Fig. 7A). When we incubated
membranes with [35S]GSSG for short time periods complex I
 glutathionylation only occurred in the presence of Grx2.

Functional Consequences of Glutathionylation on Complex I
Activity—The extensive glutathionylation of complex I cata-
yzed by Grx2 raises several interesting questions. The identity
of the cysteine residues glutathionylated and the relationship
between complex I glutathionylation and mitochondrial ROS
production (42) will be reported elsewhere. Here we have ex-
plored how glutathionylation and incubation with an oxidized
 glutathione pool affect complex I enzyme activity. To do this we
measured the NADH-ubiquinone oxidoreductase activity and
the NADH-linked respiration rate of mitochondrial membranes
in the presence of saturating NADH concentrations. Preincuba-
tion with 5 mM GSSG ± Grx2 for 3 min had no effect on either
measurement (data not shown). There was also no effect of
GSSG ± Grx2 on the apparent Km for NADH of either NADH-
ubiquinone oxidoreductase activity, or on the NADH-linked
respiration rate (data not shown). As the glutathionylation of com-
plex I occurs readily under these conditions (Figs. 4–7),
glutathionylation alone does not affect the activity of complex I.

As prolonged incubation with supraphysiological GSSG con-
centrations disrupts complex I (42), we next determined how
complex I activity was affected by incubating mitochondrial
membranes with physiological GSH/GSSG ratios (Fig. 8). In-
cubation with 5 mM GSSG led to the gradual but extensive
inactivation of complex I (Fig. 8A). The effect of 10 mM GSH
was indistinguishable from controls, while incubation with 5
mM GSH/2.5 mM GSSG led to an intermediate rate of inactiva-
tion (Fig. 8A). Therefore complex I inactivation depends on the
GSH/GSSG ratio. Complex I was more susceptible to inactiva-
tion by GSSG than other respiratory complexes (Fig. 8B). When
the incubation shown in Fig. 8A was repeated under argon, or
in the presence of NADH or succinate, the rate of inactivation
was unchanged indicating that neither nonspecific oxidative
damage nor the reduction state of the respiratory chain con-
tribute (data not shown). Grx2 did not affect the rate of inac-
tivation by 5 mM GSSG, but at lower GSSG concentrations
Grx2 significantly accelerated complex I inactivation, bringing
about similar levels of inactivation as seen for 5 mM GSSG (Fig.
8C). Inhibition of complex I by incubation with relatively re-
duced GSH/GSSG ratios was not affected by Grx2, indicating
that the inactivation of complex I was a function of the GSH/
GSSG ratio and not the GSSG concentration (Fig. 8D). Incuba-
tion of membranes with 5 mM of the simple disulfides HEDS or
cystine for 1 h had similar inhibitory effects on complex I as 5
mM GSSG, whereas incubation with 100 μM GSSG, cystine or
HEDS did not affect complex I activity (data not shown). How-
ever, in the presence of Grx2 100 μM GSSG inhibited complex
I (Fig. 8C), while the inhibition by cystine and HEDS were
unaffected by Grx2 (data not shown). Therefore incubation
with high concentrations of disulfides inhibits complex I, how-
ever low concentrations of GSSG are particularly effective at
inhibiting complex I in the presence of Grx2.

The inhibition of complex I by 5 mM GSSG was only partially
reversed by deglutathionylating complex I with DTT, GSH, or
GSH ± Grx2 (Fig. 8E). Inactivation of complex I by treatment
with 100 μM GSSG in the presence of Grx2 was also only
partially reversed by DTT and GSH ± Grx2 (data not shown).
Therefore maintenance of complex I glutathionylation or thiol
oxidation was not essential to sustain the loss in complex I
activity. To see if complex I inactivation required the continual
presence of GSSG, or if an initial interaction with GSSG was
sufficient, we incubated mitochondrial membranes ± GSSG for
30 min. The activity of complex I was then measured and the
membranes were reincubated ± GSSG (arrow, Fig. 8F). The
complex I activity was then assessed over time (Fig. 8F).
The inactivation of complex I depended on the presence of an
oxidized glutathione pool, as the rate of inactivation in the pres-
ence of GSSG returned to the baseline level once the GSSG was
removed: conversely, the rate of inactivation of complex I that
had not been exposed to GSSG increased once GSSG was present
(Fig. 8F). However, when the extent of glutathionylation of com-
plex I was assessed on immunoblots, it was found that complex I
remained glutathionylated for at least an hour after removal of the GSSG although the extent of glutathionylation was less than that of membranes incubated with GSSG (data not shown). Therefore the loss of complex I activity did not simply correlate with the glutathionylation of the enzyme. Instead, the loss of complex I activity is a consequence of long term maintenance of the complex in the presence of an oxidized glutathione pool.

DISCUSSION

Here we have investigated the interplay between mitochondrial membrane protein thiols and the glutathione pool. There are about 35 nmol/mg protein reactive protein thiols exposed on the surface of mitochondrial membranes, comprising about 40% of the total protein thiols. Thus the concentration of exposed protein thiols within the mitochondrial matrix is greater than that of GSH, suggesting that the interaction between the glutathione pool and protein thiols plays a critical role in mitochondrial antioxidant defense. Up to ~70% of the exposed protein thiols react with GSSG with some responding rapidly, within a minute or so, while the remaining protein thiols change their redox state gradually over 30 min. The more oxidized the glutathione pool the greater the thiol oxidation, although about 30% of the protein thiols were not oxidized by GSSG over 30 min. The wide range of protein thiol reactivity is probably due to variations in thiol pKa and accessibility (27) and is important for both the antioxidant and redox signaling roles of protein thiols. This is because a range of reactivities enables some thiol proteins to respond dramatically to small changes in the GSH/GSSG ratio whereas others will only start to interact with the glutathione pool under conditions of extreme oxidative stress. The mitochondrial thiol proteins that remained glutathionylated for at least an hour after removal of the GSSG although the extent of glutathionylation was less than that of membranes incubated with GSSG (data not shown). Therefore the loss of complex I activity did not simply correlate with the glutathionylation of the enzyme. Instead, the loss of complex I activity is a consequence of long term maintenance of the complex in the presence of an oxidized glutathione pool.

FIG. 8. Functional consequences of glutathionylation of complex I. Respiratory complex activities are the inhibitor-sensitive rates measured in duplicate and expressed as a percentage of untreated controls at time 0, and are mean ± range of a typical experiment repeated 2–3 times. A, loss of complex I activity over time. Mitochondrial membranes were incubated as described in the legend to Fig. 1A with no additions, 10 mM GSH, 2.5 mM GSSG/5 mM GSH, or 5 mM GSSG. B, effect of GSSG on respiratory complex activity. Mitochondrial membranes were incubated with 5 mM GSSG for 60 min as in A. The activities of complex I, complexes II/III, and complex IV are shown. C, effect of Grx2 on complex I inactivation by a range of concentrations of GSSG. Mitochondrial membranes were incubated with various concentrations of GSSG ± Grx2 for 60 min as in A and the effect on complex I activity measured. The inset shows a log plot of the same data. D, effect of Grx2 on complex I inhibition by various GSH/GSSG ratios. Mitochondrial membranes were incubated with various GSH/GSSG ratios ± Grx2 for 60 min as in A, and the effect on complex I was measured. E, limited reversal of complex I inactivation. Mitochondrial membranes were incubated as in A for 60 min ± 5 mM GSSG, and samples were assessed for activity at that time. The membranes were then incubated for a further 30 min with either no additions, 1 mM DTT, or 10 mM GSH ± Grx2. F, dependence of GSSG-induced decay on duration of GSSG exposure. Mitochondrial membranes were incubated for 30 min ± 5 mM GSSG then (arrow) both sets of membranes were incubated for a further 90 min ± 5 mM GSSG and complex I activity measured over time.
respond particularly sensitively to changes in GSH/GSSG ratio are now being identified by proteomic approaches using mitochondria-targeted thiol protein reagents (32, 43). A wide range of mitochondrial membrane proteins contain exposed, reactive thiols that reacted with GSSG by thiol-disulfide exchange to form a mixed disulfide (Reaction 1). However, only a few thiol proteins remained glutathionylated, with most displacing the GSH to form an intraprotein disulfide (Reaction 2). Consequently, the proportion of protein thiols that was persistently glutathionylated was far smaller than that which formed intraprotein disulfides, with complex I standing out as a persistently glutathionylated mitochondrial membrane protein. The mechanistic reason for greater intraprotein disulfide formation is that most glutathionylated protein thiols are formed adjacent to a second thiol that rapidly displaces GSH to form an internal disulfide (Reaction 2). 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. An important function for changes in the oxidation state of protein thiols is in redox sensing and signaling (2–4). This occurs when the reversible oxidation or reduction of a protein thiol allows it to change its function in response to alterations in the GSH/GSSG ratio. Protein activity can change following formation of an intraprotein disulfide, as happens for the transcription factor OxyR (25), or by formation of a protein-glutathione mixed disulfide, as occurs with carbonic anhydrase (44). However, for these changes in protein activity to function as redox switches, the ratio of oxidatively modified to unmodified protein must change appropriately in response to an altered GSH/GSSG ratio (2–4). For the formation of a protein-mixed disulfide (PrS-SG) the equilibrium is shown in Equation 2.

\[ K_1 = \frac{[\text{PrS-SG}][\text{GSH}]}{[\text{PrSH}][\text{GSSG}]} \] (Eq. 2)

In contrast, for the formation of an intraprotein disulfide (PrS2), the equilibrium is shown in Equation 3.

\[ K_2 = \frac{[\text{PrS}_2][\text{GSH}][\text{GSSG}]}{[\text{Pr(SH)}_2]} \] (Eq. 3)

Hence the Pr(SH)/PrS2 ratio is proportional to [GSH] whereas the PrSH/PrSSG ratio is proportional to [GSH] as in Equations 4 and 5.

\[ \frac{[\text{PrS-SG}]}{[\text{PrSH}]} = K_1[\text{GSSG}]/[\text{GSH}] \] (Eq. 4)

\[ \frac{[\text{PrS}_2]}{[\text{Pr(SH)}_2]} = K_2[\text{GSSG}]/[\text{GSH}] \] (Eq. 5)

Therefore the same change in the GSH/GSSG ratio will cause a significantly greater alteration in the PrSH/PrS2 ratio compared with that in the PrSH/PrS-SG ratio (2–4). 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 (1, 2). A corollary is that formation of the mixed disulfide is only affected by the GSH/GSSG ratio, while the PrSH/PrS2 ratio is also affected by [GSH]. Such subtle differences may be important when the [GSH] changes, for example, during GSH efflux from apoptotic cells (45). Another important function of protein thiols is in buffering the GSH/GSSG ratio during transient oxidative stress. The formation of a protein disulfide converts GSSG to two GSH molecules, whereas formation of a mixed disulfide generates only one GSH, and depletes glutathione equivalents by leaving a GSH bound to protein. Therefore the formation of intraprotein disulfides may facilitate buffering of the mitochondrial GSH/GSSG ratio by protein thiols. Future work should indicate whether these subtle differences in the response of protein thiols to the GSH/GSSG ratio can help explain why most protein thiols form intraprotein disulfides rather than persistent protein-glutathione mixed disulfides.

The oxidation and persistent glutathionylation of protein thiols by GSSG were catalyzed dramatically by Grx2, even at relatively reduced GSH/GSSG ratios. Furthermore, the deglutathionylation of protein-glutathione mixed disulfides and the reduction of protein disulfides were also catalyzed by Grx2. Thus Grx2 stands at the center of the reversible interactions of protein thiols with the mitochondrial glutathione pool (Scheme 1). The very rapid response of protein thiols in the presence of...
Grx2 to slight oxidations of the GSH/GSSG ratio will enable protein thiols to transduce redox signals rapidly and reversibly into changes in protein activity. By speeding up thiol-disulfide exchange, Grx2 will greatly facilitate the buffering of the GSH/GSSG ratio in mitochondria by protein thiols. In addition, Grx2 specifically catalyzes the removal of GSH from glutathionylated mitochondrial membrane proteins, extending earlier studies, which showed that Grx2 could de-glutathionylate model glutathionylated proteins (31). This reversal of glutathionylation is important for enabling the reversibility of protein thiol changes during redox signaling and also in facilitating the antioxidant role of mitochondrial protein thiols. In degrading ROS, protein thiols will form thyl radicals and sulfenic acids which can react rapidly with GSH to form mixed disulfides (Reactions 5 and 6), thereby preventing further protein oxidation. The rapid de-glutathionylation of these mixed disulfides by Grx2 will rapidly restore the protein thiols and supports a role for exposed protein thiols in mitochondrial antioxidant defense.

It was of particular interest that complex I stood out as one of the very few mitochondrial membrane proteins to be persistently glutathionylated. Complex I is a large mitochondrial inner membrane protein of ~1 MDa that contains 46 polypeptide subunits, a flavin mononucleotide (FMN) cofactor and a number of iron-sulfur centers (46). Its principal role is as an NADH-ubiquinone oxidoreductase that is coupled to proton pumping across the mitochondrial inner membrane and which acts as a gateway for electrons into the respiratory chain (46). It was clear that glutathionylation itself does not lead to a direct alteration of the activity of the complex. Instead the duration of exposure to an oxidized GSH/GSSG ratio was critical for inactivation. 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 (47, 48).

In addition to its action as a redox coupled proton pump, complex I is a major source of ROS within the cell (49), is involved in the mitochondrial permeability transition (50), is particularly interesting that the 75 and 51 kDa subunits of complex I were the ones glutathionylated, as these are the entry site for electrons from NADH into complex I (46). In addition, the selective cleavage of the 75 kDa subunit by caspases is an important early event in apoptosis (51), therefore it is tempting to speculate that the Grx2-catalyzed glutathionylation of the 75 kDa subunit is related to committing cells to apoptosis. Future work will investigate whether the well established link between oxidation of the mitochondrial glutathione pool and apoptosis is related to the glutathionylation of complex I.

In summary, we have shown that 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 changes in the GSH/GSSG ratio during oxidative damage and redox signaling (Scheme 1).

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Glutaredoxin 2 Catalyzes the Reversible Oxidation and Glutathionylation of Mitochondrial Membrane Thiol Proteins: IMPLICATIONS FOR MITOCHONDRIAL REDOX REGULATION AND ANTIOXIDANT DEFENSE

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