Glutaredoxin 2 Catalyses the Reversible Oxidation and
Glutathionylation of Mitochondrial Membrane Thiol Proteins:
Implications for Mitochondrial Redox Regulation and Antioxidant Defence

Samantha M. Beer, Ellen R. Taylor, Stephanie E. Brown, Christina C. Dahm,
Nikola J. Costa, Michael J. Runswick, and Michael P. Murphy*

From the Medical Research Council Dunn Human Nutrition Unit, Wellcome Trust-
MRC Building, Hills Road, Cambridge CB2 2XY, UK

*To whom correspondence should be addressed: Dr Michael P. Murphy, MRC Dunn
Human Nutrition Unit, Wellcome Trust-MRC Building, Hills Road, Cambridge CB2
2XY, UK. Fax +44-1223-252905; E-mail: mpm@mrc-dunn.cam.ac.uk

Running title: Glutaredoxin 2 and Mitochondrial Protein Thiols
The redox poise of the mitochondrial glutathione pool is central in the response of mitochondria to oxidative damage and redox signalling, 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 oxidised 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 minutes. 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 catalysed 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 oxidised 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 catalysed reversible protein glutathionylation/deglutathionylation over a wide range of GSH/GSSG ratios, from the reduced levels accessible under redox signalling to oxidised 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 signalling 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 signalling reactive oxygen species (ROS) oxidise GSH to GSSG directly, or catalysed 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 (1)) (Reaction 1).

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

**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 (Reaction 2) (1).

$$\text{Pr} \begin{array}{c} \text{S-SG} \\ \text{S}^- \end{array} \rightarrow \text{Pr} \begin{array}{c} \text{S} \\ \text{S} \end{array} + \text{GS}^-$$

**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 (Reactions 3 & 4), although protein disulfides can also be reduced by thioredoxin 2 (Trx2) (7,8) and glutaredoxin 2 (Grx2) (9,10).

$$\text{Pr} \begin{array}{c} \text{S} \\ \text{S} \end{array} + \text{GSH} \rightarrow \text{Pr} \begin{array}{c} \text{S-SG} \\ \text{SH} \end{array} \rightarrow \text{Pr} \begin{array}{c} \text{SH} \\ \text{SH} \end{array} + \text{GSSG}$$

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

**REACTIONS 3 & 4**

Alteration to protein thiol redox state via changes in the GSH/GSSG ratio during oxidative stress and redox signalling is thought to be particularly important in mitochondria during apoptosis, necrosis and induction of the permeability transition,
but 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 defence (2,20,21). Reactions with ROS convert protein thiols to thiy radicals or sulfenic acids, which can be further oxidised (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).

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

\[
\text{PrS}^- + \text{GSH} \rightarrow \text{PrS}^\cdash \text{SG} \quad \text{H}^+ \quad \text{O}_2 \quad \text{O}_2^\cdash
\]

**REACTIONS 5 & 6**

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 & 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 signalling 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 pKₐ (30). Grx catalyses 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).

Modelling 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 catalysing the interplay of the mitochondrial glutathione pool with protein thiols during both antioxidant defence and redox signalling.

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 catalysed 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 catalysed 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 defences within mitochondria.
EXPERIMENTAL PROCEDURES

Materials - (4-Iodo)butyltriphenylphosphonium iodide (IBTP) and αTPP rabbit antiserum were prepared as described (32). Rabbit antisera against the 75 kDa, 51 kDa 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. To confirm the selectivity of this antibody for glutathionylated proteins, we used BSA that had been reduced by mercaptoethanol, dialysed 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 diluting an aliquot of the [35S]GSH stock to ~50 – 70 µl in KPi buffer (50 mM KPi, 1 mM EGTA, 100 µM DTPA, pH 8) and extracting this with 4 x1 ml CHCl₃. Ferrocytochrome c was prepared by reduction of bovine heart cytochrome c (Sigma) with excess dithionite followed by gel filtration on Sephadex G-25.

Preparation and Incubation of Mitochondria, Membranes and Complex I - Bovine heart mitochondria were prepared as described (33). 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 less than 10 % citrate synthase specific activity in membrane preparations 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 a concentration range (0.001 - 0.1% w/v) which completely 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 protein/ml with 1 mM DTT in KPi buffer for 10 min at 37°C. The membranes were then pelleted by centrifugation (15,000 x g) and washed in KPi buffer. Complex I was prepared by solubilisation of membranes with dodecyl-β-D-maltoside (DDM, Antrarce, OH) followed by ion-exchange chromatography (35). Pooled fractions were further purified by ion-exchange separation, ammonium sulfate precipitation and gel filtration, and the pure complex I was stored in buffer containing 0.1 % DDM and 10 % ethylene glycol at –80°C.

Thiol Assays - Two assays were used to measure exposed protein thiols: the dithionitrobenzoic acid (DTNB; Ellman's reagent) assay and the papain assay. To measure exposed protein thiols by the DTNB assay, duplicate membrane samples were pelleted by centrifugation (15,000 x g for 4 min), washed once in NaPi buffer (80 mM NaPi, 1 mM EDTA, pH 8) and identical samples assessed for thiol or protein content. For thiol content the pellet was resuspended in 100 µl NaPi buffer and after pelleting the supernatant A412 was measured. The pellet was then resuspended in the same supernatant, 200 µM DTNB was added and after 30 min at ~ 23°C the membranes were pelleted, A412 of the supernatant measured, corrected for background and used to calculate thiol content ($\epsilon_{412} = 13,600$ M$^{-1}$ cm$^{-1}$ (36)). For the protein assay the pellet was treated as above in the absence of DTNB, and then resuspended in 1 ml 1% Triton-X-100 and the protein concentration measured by the bicinchoninic acid (BCA) assay using BSA as a standard (37). To measure total protein thiols by the DTNB assay membrane pellets were resuspended in NaPi buffer containing 2% SDS and 200 µM DTNB and A412 was measured in quadruplicate after 20 min incubation. Parallel samples were assessed for protein content by the BCA assay. To measure exposed protein thiols by the papain assay, duplicate membrane samples were pelleted, washed twice in ice-cold 50 mM KPi, 1 mM EGTA, 100 µM DTPA, pH 6 and once with 5 mM sodium acetate, 50 mM NaCl, 0.5 mM EDTA, pH 4.7. The
membrane pellet was then resuspended in this buffer at ~8 mg protein/ml. Free thiols were then quantified using a kit that relies on activation of papain by thiols (Molecular Probes, catalog number T-6060). For this 5 µl of sample or standard (L-cysteine; 0 – 1 nmol) were added in triplicate to a 96 well plate containing 5 µl 4 mM cystamine. Then 95 µl papain-SSCH₃ (0.6 mg/ml) was added and incubated at room temperature for 1 hour before addition of 95 µl 4.9 mM N-benzoyl-L-arginine-p-nitroanilidehydrochloride. After a further 30-60 minutes A₄₀₅ was measured using an ELx₈₀₈ ultra microplate reader (Bio-Tek Instruments Inc).

Glutathione Assays - Glutathione-protein mixed disulfides on mitochondrial membranes were measured as the amount of [³⁵S]GSH bound. For this [³⁵S]GSH (10 – 20 µCi/ml) was added to GSH/GSSG in KPi buffer to give a specific activity of 1 – 2 Ci/mol GSH. After incubation at 37°C for 30 min under argon to enable the [³⁵S]GSH to equilibrate with the GSH/GSSG, a sample (5 – 10 µl) was removed for scintillation counting to calculate the specific activity. The [³⁵S]-equilibrated GSH/GSSG solution was then incubated with mitochondrial membranes and at the end of the incubation duplicate samples (110 µl) were mixed with 50 µl ice-cold 50 mM KPi, 1 mM EDTA, 100 µM DTPA, pH 6 containing 50 mM N-ethylmaleimide (NEM). The membranes were pelleted, excess buffer removed with a tissue, washed and then resuspended and pelleted twice in this buffer without NEM. The pellet was then resuspended in 110 µl 1 % Triton and transferred to a scintillation vial by cutting off the end of the Eppendorf tube and then 3 ml scintillant (Scintran FluoranSafe 2, BDH) was added. The ³⁵S content was determined using a Packard Tri-Carb 2100TR liquid scintillation analyzer and converted to nmol GSH from the specific activity. A parallel sample was resuspended in 110 µl 1 % Triton and the protein content determined by the BCA assay using BSA as a standard (37).

For some experiments protein-glutathione mixed disulfides were also determined by the recycling assay (11). After incubation the membranes were pelleted by centrifugation, washed first in ice-cold 50 mM KPi, 1 mM EGTA, 100 µM DTPA, pH 6 and then with 5% 5-sulfosalicyclic acid containing 0.1% Triton, 0.5 mM EGTA and 1 mM DTPA. The protein pellets were then resuspended in 8 M urea (65 µl) followed by addition of 37.5 mM Tris-HCl, pH 7. Sodium borohydride (1 % w/v final) was then added, the samples vortexed and incubated at 40°C for 30 min. The proteins were precipitated with 10% sulfosalicyclic acid (15 min at room temperature,
then 15 min on ice, followed by centrifugation at 15,000 x 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 sample or standard, mixed with 295 µl 0.5 mM DTNB, 0.3 mM NADPH in 85 mM NaPi, 3.7 mM EDTA, pH 7.5 and 1 U glutathione reductase (Sigma) (11). The formation of thionitrobenzoic acid was measured at 405 nm over 10 minutes using a kinetic plate reader (ELx 808 ultra microplate 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. Gladyshev, University of Nebraska, Lincoln, USA (9). This sequence starts from the second in-frame methionine residue and lacks the mitochondrial targeting sequence and the two amino acids (SerGly) predicted to lie between the cleavage site of the mitochondrial processing peptidase and the second Met residue. The C-terminus is LeuGluHis. The construct was transformed into XL1 Blue *Escherichia coli* by electroporation using a BioRad GenePulser and purified using a Qiagen Miniprep kit. Sequencing of the plasmid (Cytomyx DNA sequencing service, Cambridge, UK) gave the sequence as reported (AF380337) (9) except for an A to C transversion at np 353. This corresponds to a His to Arg change at amino acid 106 (AAK85319.1; (9)) which is well away from both the CSYC active site and the predicted glutathione binding site. The protein sequence is:

GNSTSSFWGKSTTPVNQETISNNCVVIFSKTSCYSMAKKIFHDMNVNY
KAVELDMLEYGNYFDQDALRKMTGERTVPRIFVNGRFIGGAADTHRL
HKEGKLLLPLVHQCYLKQKEERHLEHHHHH, pI 9.12, Mw 14,995 Da.

The construct was transformed into C41(DE3) *E. coli* and grown on 2x TY medium plus ampicillin to A$_{600}$ = 0.6, then 0.6 mM isopropyl-1-thiol-β-D-galactopyranoside was added and the cells were incubated overnight at 25°C, harvested by centrifugation, resuspended in 30 ml 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9, using a Dounce homogeniser and lysed using a French Pressure cell (Slm-Aminco Spectronic Instruments). The crude lysate was centrifuged at 12,000 x g for 10 min at 4°C and the supernatant was centrifuged again at 160,000 x g for 3 hours at 4°C. The supernatant from the second centrifugation was applied to a 20 ml Ni-NTA Superflow column (Qiagen Cat No. 30210) pre-equilibrated with 0.5 M NaCl, 20 mM
Tris-HCl, pH 7.9. The protein was then eluted using an imidazole gradient from 0–1 M run over 100 min. Grx2 eluted at [imidazole] ~ 0.45 M. Pooled fractions were dialysed overnight against 1 mM KPi, 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 KPi, pH 7.5 at 2.5 – 5 mg protein/ml. Grx2 enzyme activity was measured in 1 ml 0.1M KPi, pH 7.4 containing 0.2 mM NADPH, 0.7 mM β-hydroxyethylene disulfide (HEDS), 0.5 mM GSH and 0.4 U/ml glutathione reductase (9). After preincubation at 30°C for 10 min, the background rate was recorded for 2 min, then 0-10 µg Grx2 was added and the loss of NADPH followed at 340 nm. The slope of the linear portion of the time course was corrected for background and the activity calculated ($\varepsilon_{340} = 6,220 \text{ M}^{-1} \cdot \text{cm}^{-1}$). Grx2 gave a specific activity of 7–9 U/mg protein consistent with published values (9, 10) (1 U = 1 µmol NADPH/minute). 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 rapidly thawed, stored on ice and discarded after use. Grx2 was used at 1 U/ml for all experiments.

_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% sulfosalicylic acid (SSA) 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 Protean system and transferred to PVDF overnight at 4°C using a Bio-Rad Mini Protean Transfer Cell. The blot was incubated with antiserum followed by a secondary antibody-horseradish peroxidase conjugate and visualised by enhanced chemiluminescence (ECL; Amersham Pharmacia Biotech). 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 protein was resuspended in 100 µl extraction buffer (0.75 M aminocaproic 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 x g). The supernatant was mixed with 5% Serva Blue in 500 mM aminocaproic acid, loaded on a 5-12% or 5-15% gradient gel in a Bio-Rad Mini Protean system and run overnight at 4°C with ferritin as a molecular weight marker (39). For fluorography of [35S]-labelled proteins, the BN-PAGE gel was fixed and stained in Coomassie Blue, soaked in Amplify (Amersham Pharmacia Biotech), 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 analysed 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 programme (http://www.matrixscience.com) was used for database 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 (ε340 = 6,220 mM⁻¹.cm⁻¹) in KPi 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 KPi buffer supplemented with 20 mM succinate, 2 mM KCN and 4 µg/ml rotenone. After 5 min pre-equilibration 30 µM ferricytochrome c was added, and its rate of reduction was measured (ε550 = 21,000 M⁻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 (ε520 = 6.28 mM⁻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 (41). The KCN-insensitive rate was negligible. Respiration rate of membranes was measured using a Clark type oxygen electrode (Rank Brothers, Bottisham, Cambridge, UK). For this, membranes (1 mg 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 $87 \pm 5 \text{ nmol thiol/mg protein}$ (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 (Fig 1A). There were about 25 nmol thiol/mg protein exposed in native membranes which increased to ~35 nmol thiol/mg protein on pretreatment with the thiol reductants DTT or GSH, due to reduction of accumulated disulfides and sulfenic acids (Fig 1A). All these protein thiols were accessible in native membranes, as the membrane impermeant 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 interact with the glutathione pool.

Incubation of Mitochondrial Membranes with Glutathione Disulfide (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 ($pK_a$ 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/2GSH reduction potential is dependent on [GSH]$^2$ as is shown in Equation 1, which gives the $E_{h,8}$ value at 25°C. This value
was derived from $E^{\circ} = -240$ mV at 25°C (2,3); the $E_{n,8}$ values at 37°C are likely to be marginally higher.

$$E_{n,8} \text{ (mV)} = -299.1 - 29.6 \log_{10}([\text{GSH}]^2/[\text{GSSG}])$$  \hspace{1cm} (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 oxidised 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 incubation with 5 mM GSSG, only about 60–70% of the exposed thiols were oxidised. 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 oxidised glutathione pool reacts with the protein thiols by thiol-disulfide exchange with GSSG (Reaction 1), and not by forming protein thyl radicals or sulfenic acids (Reactions 5 & 6).

*Incubating Mitochondrial Membranes with GSSG Leads to Limited Persistent Glutathionylation* - The loss of exposed protein thiols in Figs 1B 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 $[^{35}\text{S}]$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 $[^{35}\text{S}]$-equilibrated GSH/GSSG ratios for 1 min or 30 min and the amount of membrane-bound $[^{35}\text{S}]$GSH quantitated by scintillation counting (Fig 1E). The amount of membrane-bound GSH increased with time and with oxidation of the GSH/GSSG ratio, and the maximum amount bound was ~ 6–8 nmol GSH/mg protein.
Displacing protein-bound GSH with borohydride, followed by measurement of released GSH by the recycling assay, gave similar results (data not shown). Comparison of Figs 1B and D shows that while 5 mM GSSG oxidises ~ 25 nmol thiol/mg protein of the 35 nmol thiol present, only ~ 7 nmol glutathione/mg protein bound to the membranes. Therefore on incubation with 5 mM GSSG ~ 50% exposed protein thiols are oxidised to protein disulfides, ~ 20% form persistent mixed disulfides with GSH and ~ 30% are not oxidised.

Glutaredoxin 2 (Grx2) Catalyses Protein Thiol Oxidation and Glutathionylation - To determine whether protein thiol oxidation and glutathionylation by GSSG were catalysed by glutaredoxin (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 minute incubation (Fig 2B), but when the incubation was extended to 30 minutes the amount of glutathione bound was the same ± Grx2 (Fig 2C). Therefore Grx2 catalyses both protein oxidation and glutathionylation by thiol-disulfide exchange with GSSG.

Grx2 Catalyses Membrane Protein Thiol Reduction and Deglutathionylation by GSH - We next determined whether Grx2 catalysed the reduction by GSH of mitochondrial protein thiols that had been oxidised by GSSG. Membranes were incubated with GSSG to generate stably oxidised 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 minutes (Fig 2D). Incubation of GSSG-oxidised membranes for 2 minutes with GSH ± Grx2 confirmed that Grx2 led to a statistically significant increase in the rate of reduction of GSSG-oxidised protein thiols by GSH (Fig 2E). To see if Grx2 catalysed deglutathionylation, 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 minute (Fig 2F). Therefore Grx2 catalyses both the deglutathionylation and the reduction by GSH of membrane protein thiols that have been oxidised by GSSG.
Qualitative Analysis of the Protein Thiols Oxidised by GSSG and Grx2 - To complement the quantitation of thiol oxidation by GSSG, we visualised membrane proteins whose thiols had been altered by GSSG. This was done by labelling protein thiols with tags that can be detected on immunoblots (Fig 3A). Maleimide-biotin (MAL) reacted rapidly with all protein thiols; in contrast, both iodoacetamide-biotin (IAM) and 4-iodobutyltriphenylphosphonium (IBTP) reacted slowly, labelling reactive thiols preferentially (32). Preincubating the membranes with GSSG/Grx2 completely blocked IBTP and IAM binding, but only slightly decreased MAL labelling, 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 protein thiols, while IBTP and IAM only decreased free thiols to 96 ± 7% and 92 ± 4% of controls respectively (mean ± sd, n = 3). Therefore IBTP and IAM label a reactive subset of the exposed protein thiols in mitochondrial membranes that is also particularly reactive with GSSG. The pattern of protein labelling by IBTP and MAL indicates that reactive thiols are present on many mitochondrial membrane proteins.

Exposure of mitochondrial thiol proteins to GSSG/Grx2 could lead to interprotein disulfide formation and subsequent protein aggregation in membranes of possible physiological or pathophysiological relevance. To see if GSSG/Grx2 led to extensive protein aggregation, we compared Coomassie-stained gels of membrane proteins that had been exposed to GSSG or GSSG/Grx2 and separated out by non-reducing SDS-PAGE (Fig 3B). The sample buffer contained NEM to block thiols exposed on SDS treatment and thereby prevent artefactual protein crosslinking during sample preparation. The protein distribution was not grossly affected by GSSG ± Grx2 as indicated by retention of all major protein bands and the lack of aggregation or smearing of bands (Fig 3B, -DTT). In addition, parallel samples treated with DTT were similar to those run under non-reducing conditions (Fig 3B, +DTT). These data make it unlikely that there is extensive interprotein disulfide formation between abundant mitochondrial thiol proteins on exposure to GSSG/Grx2, however interprotein crosslinks between non-abundant proteins will not be detected by this procedure. Therefore the formation of protein disulfides caused by GSSG/Grx2 is predominantly due to intraprotein disulfides.
**Visualisation of Persistently Glutathionylated Proteins** - A proportion of membrane protein thiols should be stably glutathionylated following incubation with GSSG (Figs 1 & 2). To visualise 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 stabilise protein-glutathione mixed disulfides and prevent the artefactual 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 labelled by IBTP or IAM (Fig 3A), in agreement with the measurements in Fig 1.

**Grx2 Catalyses Protein Glutathionylation at Relatively Reduced GSH/GSSG Ratios** - The ready visualisation 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 seconds 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 seconds (Fig 4E). This confirms that Grx2 catalyses 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 thioredoxin (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 2D gels. Finally, we confirmed that isolated complex I was stably glutathionylated on the 75 and 51 kDa subunits on exposure to GSSG (Fig 6A). Therefore the predominant mitochondrial membrane proteins that are persistently glutathionylated by GSSG are the 75 and 51 kDa subunits of complex I.

Grx2 Catalyses Glutathionylation of Complex I - Grx2 dramatically catalysed 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 catalysed 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 GSSG alone over 1 minute, 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 glutathionylation even at quite reduced GSH/GSSG ratios (Fig 6B). At the more
oxidised GSH/GSSG ratios Grx2 catalysis was less evident, presumably due to rapid spontaneous glutathionylation under these conditions.

To demonstrate further that Grx2 catalysed the glutathionylation of complex I, we incubated mitochondrial membranes with [³⁵S]GSSG and assessed complex I glutathionylation by fluorography of BN-PAGE gels (Fig 7A). Long-term incubation of mitochondrial membranes with GSSG led to complex I glutathionylation 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 catalysed 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 explored how glutathionylation and incubation with an oxidised 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. Preincubation with 5 mM GSSG ± Grx2 for 3 minutes had no effect on either measurement (data not shown). There was also no effect of GSSG ± Grx2 on the apparent $K_m$ for NADH of either NADH-ubiquinone oxidoreductase activity, or on the NADH-linked respiration rate (data not shown). As the glutathionylation of complex 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 concentrations disrupts complex I (42), we next determined how complex I activity was affected by incubating mitochondrial membranes with physiological GSH/GSSG ratios (Fig 8). Incubation 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 inactivation (Fig 8A). Therefore complex I inactivation depends on the GSH/GSSG ratio. Complex I was more susceptible to inactivation 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 non-specific oxidative damage nor the reduction state of the
Grx2 did not affect the rate of inactivation 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 reduced 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). Incubation of membranes with 5 mM of the simple disulfides HEDS or cystine for 1 hour had similar inhibitory effects on complex I as 5 mM GSSG, while incubation with 100 µM GSSG, cystine or HEDS did not affect complex I activity (data not shown). However, 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, however 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 were 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 assayed over time (Fig 8F). The inactivation of complex I depended on the presence of an oxidised glutathione pool, as the rate of inactivation in the presence 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 complex 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 oxidised 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 defence. 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 minutes. The more oxidised the glutathione pool the greater the thiol oxidation, although about 30% of the protein thiols were not oxidised by GSSG over 30 minutes. The wide range of protein thiol reactivity is probably due to variations in thiol pK_a and accessibility (27) and is important for both the antioxidant and redox signalling 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 while others will only start to interact with the glutathione pool under conditions of extreme oxidative stress. The mitochondrial thiol proteins that 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 defence to favour 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 signalling (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:

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

In contrast, for the formation of an intraprotein disulfide (PrS₂) the equilibrium is:

\[ K_2 = \frac{[PrS_2][GSH]^2}{[Pr(SH)_2][GSSG]} \] (Eq. 3)

Hence the Pr(SH)₂/PrS₂ ratio is proportional to [GSH]² while the PrSH/PrSSG ratio is proportional to [GSH].

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

\[ \frac{[PrS_2]}{[Pr(SH)_2]} = K_2 \frac{[GSSG]}{[GSH]^2} \] (Eq. 5)

Therefore the same change in the GSH/GSSG ratio will cause a significantly greater alteration in the PrSH/PrS₂ ratio compared to that in the PrSH/PrSSG 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, while 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 glutathione-protein mixed disulfides.

The oxidation and persistent glutathionylation of protein thiols by GSSG were catalysed 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 catalysed 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 catalyses the removal of GSH from glutathionylated mitochondrial membrane proteins, extending earlier studies which showed that Grx2 could deglutathionylate model glutathionylated proteins (31). This reversal of glutathionylation is important for enabling the reversibility of protein thiol changes during redox signalling and also in facilitating the antioxidant role of mitochondrial protein thiols. In degrading ROS, protein thiols will form thiol radicals and sulfenic acids which can react rapidly with GSH to form mixed disulfides (Reactions 5 & 6), thereby preventing further protein oxidation. The ready deglutathionylation of these mixed disulfides by Grx2 will rapidly restore the protein thiols and supports a role for exposed protein thiols in mitochondrial antioxidant defence.

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
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 oxidised 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 susceptible to inactivation during degenerative diseases and other pathologies (47,48) and is also involved in early mitochondrial changes during apoptosis (51). The central role of complex I in mitochondria suggest that the Grx2-catalysed glutathionylation of complex I in response to slight oxidation of the mitochondrial glutathione pool may have physiological significance. It 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-catalysed 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 catalysed by Grx2, enabling protein thiols to respond rapidly to changes in the GSH/GSSG ratio during oxidative damage and redox signalling (Scheme 1).

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Footnote

1The abbreviations used are: BCA, bicinchoninic acid; DDM, dodecyl-β-D-maltoside; DTPA, N,N-bis(2 bis[carboxymethyl] aminoethyl) glycine; DTNB, dithionitrobenzoic acid; DTT, dithiothreitol; FCCP, carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone; FMN, flavin mononucleotide; GR, glutathione reductase; GSH, glutathione; αGSH, antiserum against glutathionylated protein; GSSG, glutathione disulfide; Grx, glutaredoxin; HEDS, β-hydroxyethylene disulfide; high performance thin layer chromatography; IAM, iodoacetamide-biotin; IBTP, (4-iodo)butyltriphenylphosphonium iodide; MAL, maleimide-biotin; MBTA, 4-(N-maleimido) benzyl-α-trimethylammonium iodide; MTSET, [2-(trimethylammonium)ethyl]methanethiosulfonate bromide; NEM, N-ethylmaleimide; PrSH, protein thiol; PrS-SG, protein glutathione mixed disulfide; PrS₂, intraprotein disulfide; SSA, sulfosalicylic acid; TPMP, methyltriphenylphosphonium cation; TPP, triphenylphosphonium cation; αTPP, antiserum against the TPP cation; Trx, thioredoxin; TrxR, thioredoxin reductase.

REFERENCES


**Figure legends**

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Fig. 1. Effect of glutathione disulfide (GSSG) on mitochondrial membrane protein thiols. A, Quantitation of reactive membrane protein thiols. Mitochondrial membranes pretreated with DTT (filled bars) were incubated at 0.5 mg protein/ml under argon at 37°C in KPi buffer with either no additions, 100 µM NEM (N-ethylmaleimide), 250 µM MTSET ([2-(trimethylammonium)ethyl]methanethiosulfonate bromide) or 250 µM MBTA (4-(N-maleimido)benzyl-α-trimethylammonium iodide) 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 thiol/mg 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 ± SD of three experiments. D, Redox equilibration of [35S]GSH with GSH and GSSG. [35S]GSH (5 µCi/ml) was added to 10 mM GSH, 5 mM GSH/2.5 mM GSSG or 5 mM GSSG in KPi buffer and incubated at 30°C under argon for 30 min. Then 1 µl samples were spotted onto a HPTLC plate and developed in butanol:acetic acid:water (3:2:2) for 1.5 hours, dried and the radioactivity visualised using a Packard Cyclone phosphorimager. The Rf values for GSH and GSSG under these conditions were 0.64 and 0.42, respectively, in agreement with the radiolabelled bands. E, Quantitation of [35S]GSH bound to mitochondrial membranes. KPi buffer supplemented with 10 mM GSH/1 mM NADPH/0.4 U/ml glutathione reductase (GR), 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 $[^{35}\text{S}]\text{GSH}$. Data are means ± ranges for two independent experiments.

Fig. 2. **Effect of glutathione disulfide (GSSG) and Glutaredoxin2 (Grx2) on mitochondrial membrane protein thiols.** A, Loss of mitochondrial membrane protein thiols by GSSG ± Grx2. Mitochondrial membranes were suspended as in Fig 1A with 5 mM GSSG ± Grx2 and the thiols assayed by the papain assay. Data are a percentage of a 5 min incubation with 10 mM GSH and are means ± SEM of three experiments; *p < 0.05 by Student's t test for paired data. The protein thiol content was the same ± Grx2 after 15 min incubation (data not shown). B, Stimulation of $[^{35}\text{S}]\text{GSH}$ binding to mitochondrial membranes by Grx2. DTT-treated mitochondrial membranes were incubated as in Fig 1E with 5 mM GSSG ± Grx2, or 10 mM GSH ± Grx2 and the amount of membrane-bound $[^{35}\text{S}]\text{GSH}$ was determined. Data are means ± SEM 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 $[^{35}\text{S}]\text{GSH}$ 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 of two independent experiments. E, Reversibility of thiol loss by GSH and Grx2 measured by the papain assay. Mitochondrial membranes were incubated as in Fig 1A with 5 mM GSSG and Grx2 for 10 minutes, 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 U/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 DTT treatment and are means ± SEM of seven experiments. *p < 0.05 by Student's t test for paired data. F, Reversal of $[^{35}\text{S}]\text{GSH}$ binding to membranes by Grx2 and GSH. KPi buffer containing 5 mM GSSG was preincubated with 38 μCi/ml $[^{35}\text{S}]\text{GSH}$ 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 in 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.

FIG. 3. **Visualisation of thiol oxidation of mitochondrial membrane protein thiols.**

A, Mitochondrial membrane protein thiols oxidised by GSSG. Mitochondrial membranes were incubated as in Fig 1A with no additions or with 5 mM GSSG ± Grx2 for 5 min. Membrane samples (100 µg protein) were then pelleted and resuspended in 100 µl KPi buffer containing 100 µM maleimide-biotin (MAL), iodoacetamide-biotin (IAM) or (4-iodo)butyltriphenylphosphonium (IBTP) and incubated in the dark for 5 min at 37°C under argon. Samples (20 µg protein) were then precipitated with 10 % sulfosalicylic acid and resuspended in loading buffer supplemented with 50 mM NEM, but without DTT, separated on a 12–22 % gradient gel, blotted onto PVDF and biotin or IBTP labelling was detected. The main panel shows a single exposure, where the αMAL bands are overexposed. A shorter exposure of the MAL labelled lanes is included. B, Effect of incubation with GSSG and Grx2 on protein crosslinking. Mitochondrial membranes were incubated and prepared for SDS-PAGE as described in A in loading buffer with NEM and without DTT (-DTT), or containing 100 mM DTT (+DTT). Samples (20 µg protein) were separated on a 10–22% acrylamide SDS-PAGE gel, fixed, stained with Coomassie Blue and dried. The prominent band at ~ 15 kDa is Grx2.

FIG. 4. **Visualisation of glutathionylation of mitochondrial membrane protein thiols.** All incubations were as in Fig 1A and samples were prepared for SDS-PAGE as in Fig 3A, transferred to PVDF and detected using anti-GSH antibody. A, Visualisation of glutathionylation of mitochondrial membrane proteins. Mitochondrial membranes were incubated ± 5 mM GSSG for 5 min. A few experiments showed diffuse labelling at higher molecular weights and occasionally at around 30 kDa, but these varied for reasons that are unclear (data not shown). B, Grx2 catalysis of membrane protein glutathionylation by GSSG. Mitochondrial membranes were incubated with no additions or with 5 mM GSSG ± Grx2. C, Grx2 catalysis of membrane protein glutathionylation by GSH/GSSG. Mitochondrial membranes were
incubated with 5 mM GSH/2.5 mM GSSG ± Grx2. 

**D**, Effect of GSH/GSSG ratio and Grx2 on membrane protein glutathionylation. Mitochondrial membranes were incubated for 1 min with the indicated GSH/GSSG ratios ± Grx2. The $E_h$ values were estimated from Equation 1 which is defined for 25°C: the values at 37°C will be slightly higher. Incubation for 5 min gave a similar pattern (data not shown). 

**E**, Reversibility of membrane protein glutathionylation by Grx2. Mitochondrial membranes were incubated with 5 mM GSSG for 30 min, pelleted by centrifugation and washed. The glutathionylated membranes were then incubated with no additions, with Grx2, or with 500 µM GSH/GR (0.4 U/ml)/1 mM NADPH ± Grx2 and analysed for glutathionylation. 

**F**, Effect of thioredoxin on membrane glutathionylation. Mitochondrial membranes were incubated with no further additions (cont), 5 mM GSH/2.5 mM GSSG and Grx2 (G) or Trx (T; 1 U/ml). Trx (0.4 U/ml) was also ineffective in the presence of TrxR (2 U/ml) and NADPH (50 µM) (data not shown).

**FIG. 5** Identification of persistently glutathionylated proteins in mitochondrial membranes. 

**A**, Identification of complex I subunits by immunoblotting. Mitochondrial membranes were incubated as in Fig 1A with 5 mM GSSG ± Grx2 for 10 min and then prepared for non-reducing SDS-PAGE and 20 µg protein samples analysed for protein glutathionylation as in Fig 4. A parallel section of the immunoblot was probed with a mixture of antisera against the complex I 51 kDa and 75 kDa subunits. 

**B & C**, Identification of glutathionylation of the 75 kDa subunit of complex I. Membranes were incubated as in Fig 1A with 5 mM GSSG for 30 min and then separated by non-reducing SDS-PAGE as in A. Parallel lanes were then transferred to PVDF and immunoblotted for glutathionylated proteins (αGSH) or the gel was stained with Coomassie Blue. After alignment, the indicated band was excised and peptide mass fingerprinting performed by in-gel trypsin digestion followed by determination of the peptide masses by MALDI-ToF. The peptides that match the found masses are shown in C. The 75 kDa subunit has 727 amino acids. Twelve peptides matched predicted peptides from the bovine complex I 75 kDa subunit NP_777245, giving a sequence coverage of 24%. The second peptide was incompletely digested by trypsin.
FIG. 6. **Grx2 catalyses glutathionylation of purified complex I.** Purified complex I (5.75 µg protein) was incubated in 50 µl KPi buffer with GSSG and GSH ± Grx2 at 37°C under argon then prepared for non-reducing SDS-PAGE as in Fig 4 on 10–22% gradient gel. Immunoblots were probed with antibodies against glutathione or complex I 51 and 75 kDa subunits. A, Catalysis of glutathionylation of complex I by Grx2. Complex I was incubated with various concentrations of GSSG for 1 min. B, Effect of GSH/GSSG ratio on complex I glutathionylation. Complex I was incubated with various ratios of GSH/GSSG for the indicated times. Control: no GSH or GSSG present; +DTT: DTT was included in the loading buffer.

FIG. 7. **Grx2 catalyses glutathionylation of complex I in membranes assessed by BN-PAGE.** A, Glutathionylation of complex I. [35S]-GSH (20 µCi/ml) was preincubated with 100 µM GSSG in KPi buffer under argon for 30 min. Then mitochondrial membranes (120 µg protein) were suspended in this as in Fig 1A for 5 min in duplicate incubations + Grx2. The samples were pelleted and prepared for BN-PAGE and 120 µg protein loaded per lane. In parallel, mitochondrial membranes (80 µg protein) were incubated with non-radiolabelled GSSG and then run on the same BN-PAGE gel. The lanes containing [35S]GSH were prepared for fluorography while the unlabelled lanes were blotted onto PVDF and probed with antisera against the complex I 51 and 23 kDa subunits (αCom I). The anti-GSH antibody could also detect complex I glutathionylation on BN-PAGE immunoblots (data not shown), but the results were variable, perhaps due to inconsistent denaturation of the transferred protein. B, Stimulation by Grx2 of complex I glutathionylation. [35S]-GSH (100 µCi/ml) was preincubated with 100 µM GSSG in KPi buffer under argon for 30 min. Then mitochondrial membranes (200 µg protein) were suspended in this as in Fig 1A ± Grx2. Samples (60 µg protein) were removed at the indicated times, prepared for BN-PAGE in extraction buffer supplemented with 50 mM NEM and analysed by fluorography.
**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 zero, 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 in 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 minutes 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 minutes 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 minutes ± 5 mM GSSG and complex I activity measured over time.

**SCHEME 1.** **Grx2 catalyses reversible protein thiol glutathionylation and protein disulfide formation.** This scheme shows how Grx2 interconverts between the dithiol, glutathionylated and disulfide forms. In doing so it catalyses the glutathionylation and deglutathionylation of protein thiols and the reversible formation of protein disulfides. Grx, glutaredoxin2; GSH, glutathione; GSSG, glutathione disulfide; PrSH, protein thiol; PrS-SG, protein-glutathione mixed disulfide; TrxR, thioredoxin reductase.
Figure 3

A

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<th>αMAL</th>
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Short exposure

Long exposure

B

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M_w (kDa)

-250
-150
-75
-50
-25
-15
-10
Figure 4

A

GSSG

Mw (kDa)

B

Cont 5 mM GSSG +DTT

Grx2

Mw (kDa)

C

Cont 5mM GSH/2.5mM GSSG +DTT

Grx2

Mw (kDa)

D

[GS/][GSSG] (mM)

Eh(mV)

10/0 7.5/1.25 5/2.5 2.5/3.75

-259 -240 -217

E

Incubated with GSSG (+DTT)

GSH

Grx2

Mw (kDa)

F

Cont 5mM GSH/2.5mM GSSG +DTT

G T G T G T G T G T G T

Time (s)

300 5 15 30 60 300 300
### Figure 5

- **A**: Diagram showing protein bands with GSSG, Grx2, and DTT treatments.
- **B**: Image of a gel with a 75kDa subunit indicated.

### Table

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<th>Mr (calc)</th>
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Figure 6

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B

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Figure 7

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\(M_w\) (kDa)

880

440

B

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\(M_w\) (kDa)

880

440

Time (secs)

30

60

120
Figure 8

A

B

C

D

E

F
Scheme 1
Glutaredoxin 2 catalyses the reversible oxidation and glutathionylation of mitochondrial membrane thiol proteins: Implications for mitochondrial redox regulation and antioxidant defence
Samantha M. Beer, Ellen R. Taylor, Stephanie E. Brown, Christina C. Dahm, Nikola J. Costa, Michael J. Runswick and Michael P. Murphy

J. Biol. Chem. published online August 30, 2004

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