Specific Modification of Mitochondrial Protein Thiols in Response to Oxidative Stress

A PROTEOMICS APPROACH*

Received for publication, November 9, 2001, and in revised form, January 23, 2002
Published, JBC Papers in Press, February 22, 2002, DOI 10.1074/jbc.M110797200

Tsu-Kung Lin‡‡§§, Gillian Hughes‡‡, Aleksandra Muratovska‡‡†, Frances H. Blakie‡‡, Paul S. Brookes**‡‡, Victor Darley-Usmar**‡‡, Robin A. J. Smith‡‡, and Michael P. Murphy‡‡***

From the ‡Departments of Biochemistry and Chemistry, University of Otago, Box 56, Dunedin, New Zealand, the §Department of Neurology, Chang-Gung Memorial Hospital, Kaohsiung, Taiwan 833, the **Department of Pathology and Center for Free Radical Biology, University of Alabama, Birmingham, Alabama 35294, and †MRC-Dunn Human Nutrition Unit, MRC-Wellcome Trust Building, Hills Road, Cambridge CB2 2XY, United Kingdom

Mitochondria play a central role in redox-linked processes in the cell through mechanisms that are thought to involve modification of specific protein thiols, but this has proved difficult to assess. In particular, specific labeling and quantitation of mitochondrial protein cysteine residues have not been achieved due to the lack of reagents available that can be applied to the intact organelle or cell. To overcome these problems we have used a combination of proteomics approaches and targeted labeling of mitochondrial thiols using a novel compound, (4-iodobutyl)triphenylphosphonium (IBTP). This lipophilic cation is accumulated by mitochondria and yields stable thioether adducts in a thiol-specific reaction. The selective uptake into mitochondria, due to the large membrane potential across the inner membrane, and the high pH of the matrix results in specific labeling of mitochondrial protein thiols by IBTP. Individual mitochondrial proteins that changed thiol redox state following oxidative stress could then be identified by their decreased reaction with IBTP and isolated by two-dimensional electrophoresis. We demonstrate the selectivity of IBTP labeling and use it to show that glutathione oxidation and exposure to an S-nitrosothiol or peroxynitrite cause extensive redox changes to mitochondrial thiol proteins. In conjunction with blue native gel electrophoresis, we used IBTP labeling to demonstrate that thiols are exposed on the matrix faces of respiratory Complexes I, II, and IV. This novel approach enables measurement of the thiol redox state of individual mitochondrial proteins during oxidative stress and cell death. In addition the methodology has the potential to identify novel redox-dependent modulation of mitochondrial proteins.

Changes in the thiol redox state of mitochondrial proteins are significant in a number of cellular processes including the permeability transition, cell death due to calcium loading and oxidative stress, the response of cells to nitric oxide, tumor necrosis factor signaling, commitment to apoptosis, and in regulating respiratory chain function (1–9). However the detailed mechanisms and the proteins involved are uncertain. This is partly because of the technical challenges presented by determining thiol modifications of proteins in general and the difficulties inherent in mitochondrial proteomics. Potential protein thiol alterations include formation of mixed disulfides or internal disulfides from vicinal diithiols, S-nitrosation, and the formation of higher oxidation states (10–15). The differential reactivity of individual protein thiols and the range of lifetimes of altered redox states can act as signal sensors or transducers to influence mitochondrial function (13–17). Nitric oxide may be a particularly important regulator of mitochondrial protein thiols because it diffuses easily into mitochondria and partitions selectively into the lipid bilayer where it can modify otherwise inaccessible thiols (18–20). Modification of protein thiols by nitric oxide most likely occurs through its nitrosating derivatives peroxynitrite or NO\(^2\), and nitrosated protein thiols can go on to form mixed or internal disulfides (12). Finally, many reactive oxygen species oxidize thiols directly to thyl radicals that can then form disulfides and higher thiol oxidation states (21). In all cases the extent of the redox changes to mitochondrial thiol proteins, and the proteins affected have not been determined at the molecular level.

To identify those mitochondrial thiol proteins that change redox state during oxidative damage or cell death, we first set out to label unmodified mitochondrial protein thiols selectively within cells. Changes in protein thiol redox state would then manifest themselves by decreased labeling. To achieve this, advantage was taken of the large membrane potential across the inner membrane that causes lipophilic cations to accumulate within mitochondria (22, 23), and the lipophilic cation (4-iodobutyl)triphenylphosphonium (IBTP\(^1\)) was synthesized. It is predicted that IBTP will accumulate 5–10-fold in the cytoplasm driven by the plasma membrane potential and

The abbreviations used are: IBTP, (4-iodobutyl)triphenylphosphonium; BN-PAGE, blue native PAGE; BrBTP, (4-bromobutyl)triphenylphosphonium; CIBTP, (4-chlorobutyl)triphenylphosphonium; DTNB, dithionitrobenzoic acid; FCCP, carbonyl cyanide p-trifluoromethoxyphenylhydrazone; IEF, isoelectric focusing; NEM, N-ethylmaleimide; SNAP, S-nitroso-N-acetylpenicillamine; TBS, Tris-buffered saline; TBS, Tris-buffered saline; DMEM, Dulbecco’s modified Eagle’s medium; KLH, keyhole limpet hemocyanin; FCS, fetal calf serum; BSA, bovine serum albumin.

* This work was supported by grants from the Health Research Council of New Zealand (to M. P. M. and R. A. J. S.), the Marsden Fund, administered by the Royal Society of New Zealand (to M. P. M. and R. A. J. S.), and by National Institutes of Health Grant HL58031 (to V. D. U.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed: MRC-Dunn Human Nutrition Unit, MRC-Wellcome Trust Bldg., Hills Rd., Cambridge CB2 2XY, UK. Tel.: 44-1223-252900; Fax: 44-1223-252905; E-mail: mpm@mrc-dunn.cam.ac.uk.
Mitochondrial Thiol Proteins

should then be further concentrated several hundred-fold within mitochondria (Fig. 1) (23). Inside mitochondria protein thioliates should displace the iodo functional group of IBTP to within mitochondria (Fig. 1) (23). Inside mitochondria protein the thiolate form of a protein cysteine residue is the species that reacts with exposed protein thiols to form a stable thioether. Because the thiolate form of a protein cysteine residue is the species that reacts with exposed protein thiols to form a stable thioether, the reaction is faster at the higher pH of the mitochondrial matrix than in the cytosol.

Materials and Methods

**Chemicals**—IBTP was synthesized by reacting 1,4-diiodobutane (2.46 g; 7.9 mmol) with triphenylphosphine (0.423 g; 1.61 mmol) at 100 °C under argon in the dark for 1.5 h. The yellow solid was washed with diethyl ether (3 × 8 ml) and then dissolved in dichloromethane (5 ml) and precipitated with diethyl ether (3 × 50 ml); the solvents were decanted after each precipitation. Residual solvent was then removed under reduced pressure to give a pale yellow solid (0.518 g; 9.01 mmol; 57%) that was stored at −20 °C and protected from light. IBTP was readily soluble in ethanol giving an absorption spectrum with local maxima at 263, 268, and 275 nm and an extinction coefficient of 288 nm of 3000 M⁻¹ cm⁻¹, typical for alkyltriphenylphosphonium salts (24). For the elemental analysis,

\[ C_{30}H_{36}PI_2 \]

Calculated: C 46.18 H 4.05
Found: C 46.55 H 4.04

\[ ^1H \text{ NMR in CDCl}_3 7.7-7.9 (15H, m, Ph,CH,CH,CH,CH,Cl), 3.75-3.9 (2H, m, Ph,CH,CH,CH,CH,Cl), 3.25 (2H, t, J = 6.6 Hz, Ph,CH,CH,CH,CH,Cl), 2.28 (2H, quintet, J = 6.6 Hz, Ph,CH,CH,CH,CH,Cl), 1.82 (2H, m, Ph,CH,CH,CH,CH,Cl) 1.7-2.3 (4H, m, Ph,CH,CH,CH,CH,Cl) ppm. ^31P NMR in CDCl_3 25.2 ppm. \]

(4-Chlorobutyl)triphenylphosphonium (CBTP) was synthesized by reacting 1-chloro-4-iodobutane (0.306 g, 1.4 mmol) with triphenylphosphine (0.133 g, 0.51 mmol) and stirring at 100 °C for 1.5 h under an argon atmosphere in the dark. The mixture was cooled to room temperature, and the glassy product washed with diethyl ether (3 × 5 ml). The residue was dissolved in dichloromethane (1 ml) and precipitated with diethyl ether (10 ml), and the solvents were removed by decantation. This precipitation process was repeated three more times, and the final solid material was dried under high vacuum at room temperature to give the product (0.159 g, 0.33 mmol, 65%). ^1H NMR (CDCl_3) 7.6-7.9 (15H, m, Ph,CH,CH,CH,CH,Cl), 3.8-3.9 (2H, m, Ph,CH,CH,CH,CH,Cl), 3.70 (2H, triplet, J = 6 Hz, Ph,CH,CH,CH,CH,Cl), 2.24 (2H, quintet, J = 6 Hz, Ph,CH,CH,CH,CH,Cl), 1.85 (2H, m, Ph,CH,CH,CH,CH,Cl) ppm. ^31P NMR (CDCl_3) 25.3 ppm. CBTP was readily soluble in ethanol giving an absorption spectrum with local maxima at 263, 268, and 275 nm and an extinction coefficient of 288 nm of 3000 M⁻¹ cm⁻¹, typical for alkyltriphenylphosphonium salts (24).

(4-Bromobutyl)triphenylphosphonium (BrBTP) was from Aldrich.

**Thiol Chemistry—**BSA (1.52 mg) in 10 mM potassium phosphate (pH 7.4) was reduced with 10 mM 2-mercaptoethanol followed by dialysis against the same buffer (25) and contained ~0.64 thiol/molecule as assessed by reaction with dithionitrobenzoic acid (DTNB). The thiolamine cross-linker m-maleimidobenzoic acid N-hydroxysuccinimide ester (Sigma) and collection of the rabbit antiserum against triphenylphosphonium. For some experiments an IgG-enriched fraction was prepared using a protein A-agarose column (Affi-Gel; Bio-Rad).

SDS-PAGE gels (12.5% acrylamide) were run by standard procedures using a Bio-Rad Mini Protean system, transferred to 0.1 mm nitrocellulose using a Bio-Rad Mini Trans Blot system and blocked with 5% milk powder in TBS. Antibody binding was detected using 1:500–1:5,000 dilutions of anti-triphenylphosphonium antiserum and 1:3,000–1:10,000 dilutions of secondary antibodies (Bio-Rad) against rabbit or mouse IgG conjugated to either alkaline phosphatase and visualized by enhanced chemiluminescence.

For two-dimensional electrophoresis, mitochondria or membrane/ matrix fractions were loaded onto an Immobiline Drystrip IPI gel (Amersham Biosciences, pH range 3–10 or 4–7), and proteins were separated using the Amersham Biosciences IPIphor Isoelectric Focusing System following the manufacturer’s recommendations. The strips were then incubated in 6 μl urea, 2% SDS, 561 mM Tris-HCl, pH 8.8, 20% glycerol, 130 mM dithiothreitol for 15 min followed by incubation in 6 μl urea, 2% SDS, 561 mM Tris-HCl, pH 8.8, 20% glycerol, 135 mM iodoacetamide for 15 min, and the proteins were separated in the...
second dimension by SDS-PAGE. Gels were either stained with Coomassie Blue or blotted onto 0.1 mm nitrocellulose using a Bio-Rad Semi-dry Blotting System and probed with anti-triphenylphosphonium antiserum as described above.

Mitochondrial Incubations—Rat liver mitochondria were prepared by homogenization followed by differential centrifugation in STE medium (250 mM sucrose, 5 mM Tris-Cl, 1 mM EGTA, pH 7.4) (28), and the protein concentration was determined by the biuret assay using BSA as a standard (29). Mitochondria were subfractionated into membrane and matrix-enriched fractions by pelleting followed by incubation on ice for 30 min with the detergent Lubrol (0.2 mg/mg protein) at 10 mg of protein/ml in KCl buffer followed by centrifugation (Airfuge; 24 pounds/square inch \( \approx 100,000 \times g \) for 10 min). The matrix-enriched supernatant fraction was retained and was further concentrated by acetone precipitation (9 volumes of acetone at \(-20^\circ C\) for 30 min). The membrane fraction was washed in KCl buffer, and the protein concentration of both fractions was determined by the bicinchoninic acid assay using BSA as a standard (30).

To measure thiold content, mitochondria (1 mg of protein/ml) were incubated in KCl buffer (110 mM KCl, 10 mM Hepes, 1 mM EGTA, pH 7.2) containing 10 mM succinate and rotenone with various concentrations of IBTP at 30 °C for 15 min. To measure glutathione equivalents the mitochondria were pelleted, resuspended in 100 mM of 5-sulfosalicylic acid (5%), and glutathione equivalents quantitated by the recycling of 

\[
\text{GSH + oxidized GSH} \rightarrow \text{GSSG + } 2\text{H}^+ + 2e^-
\]

(31). Total protein thioles were determined by precipitating protein with 10% trichloroacetic acid, resuspending the pellet in 2% SDS in 80 mM sodium phosphate, pH 8, and quantitating free thioles by reaction with DTNB (32). To measure free thiols exposed in non-denatured mitochondria, the mitochondria were pelleted, resuspended in 80 mM sodium phosphate, pH 8, and disrupted by freezing in dry ice/ethanol followed by thawing in a water bath (33), and the thiol content was measured by reaction with DTNB. To measure membrane-associated free thiols in native mitochondria, a membrane fraction was isolated from the freeze-thawed mitochondria by centrifugation (13,000 \( \times g \) for 10 min) followed by washing in KCl buffer.

To measure the effects of IBTP on respiration, mitochondria (2 mg of protein/ml) were incubated at 25 °C in a 3-ml oxygen electrode in an 110 mM KCl, 10 mM Hepes, 1 mM EGTA, 1 mM potassium phosphate, pH 7.2, with either 10 mM succinate and rotenone or 5 mM each of glutamate and malate in the presence of various concentrations of IBTP. Coupled respiration was measured first, then phosphorylating respiration after addition of ADP (200 \( \mu \)M), and finally uncoupled respiration after addition of carbonylcyanide-p-trifluoromethoxyphenylhydrazone (FCCP; 200 nM). The membrane potential was measured by incubating mitochondria with 1 mM methyltriphenylphosphonium (TPMP) supplem-ented with [\( ^3H \)]TPMP (50 nCi/ml). After incubation the mitochondria were pelleted by centrifugation. The amount of TPMP in the pellet and supernatant was quantitated by scintillation counting, and the membrane potential was calculated, assuming a mitochondrial volume of 0.5 \( \mu l/mg \) protein and that 60% of intramitochondrial TPMP was membrane-bound (33). Membrane potential data were the means of triplicate determinations and were repeated on 2-3 independent mitochondrial preparations.

Mitoplasts were prepared from mitochondria by treatment with digitonin (0.125 mg of digitonin/mg of mitochondrial protein) on ice for 15 min followed by washing in STE supplemented with 0.1% fatty acid-free BSA (34). A fraction enriched for the outer membrane was prepared from the initial supernatant of the mitoplast preparation by centrifugation (144,000 \( \times g \) for 20 min) followed by washing, and the supernatant from the first centrifugation was retained as the intermembrane space fraction (34).

Cell Incubations—Human fibroblasts were grown in Dulbecco's modiﬁed Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) at 37 °C in a humidified atmosphere of 95% air, 5% CO\(_2\). Protein was quantitated by the bicinchoninic acid assay using BSA as a standard (30). For immunocytochemistry human ﬁbroblasts were plated onto 13-mm diameter glass coverslips overnight (35). Following incubation for 1 h at 37 °C with 1 \( \mu l \) IBTP \( \approx 10 \mu M \) FCCP, cells were ﬁxed with 4% paraformaldehyde in TBS for 30 min, washed with TBS, and incubated in a blocking medium (0.1% Triton-X100 in TBS, 0.1% BSA, 0.1% NaIO\(_4\)). To measure free thiols exposed in non-denatured mitochondria, a membrane fraction was isolated by centrifugation (1,500 g) and anti-cytochrome oxidase (1:100, subunit I\( a \), mouse monoclonal, Molecular Probes) diluted in TBS were then added and incubated overnight at 4 °C. The IgG fraction of preimmune serum was used as a control. After washing with TBS (3 times for 5 min), the cells were incubated with appropriate ﬂuorophore-conjugated secondary antibodies diluted in TBS, 1% Trition 20 PBS. After washing in PBS (3 times for 5 min) the pellets were dehydrated in ethanol, embedded in Lowicryl white (SPI Supplies) and polymerized under UV light at 0 °C. Ultrathin sections were mounted on nickel grids that were floated on drops of 0.1% glycine in PBS for 10 min, and 1% BSA in PBS for 15 min, followed by antipiphopholipidase antiserum overnight at 4 °C (1:10 in 1% BSA/PBS). The sections were stained with osmium tetroxide/uranyl acetate/lead citrate and examined in a CM10 transmission electron microscopy.

For immunogold electron microscopy, human fibroblasts in 24-well tissue culture plates were incubated with 200 \( \mu l \) DMEM/FCS with 1 \( \mu l \) IBTP \( \approx 10 \mu M \) FCCP for 4 h at 37 °C, then harvested with trypsin, pelleted, and washed in PBS. The pellet was warmed to 37 °C, and 200 \( \mu l \) of fixative (4% paraformaldehyde, 0.5% glutaraldehyde in 0.1 M cacodylate, pH 7.2, 6 mM CaCl\(_2\)) was added and replaced with fresh fixative after 30 s. After incubation at 37 °C for 1 h, the cell pellet was washed with 200 \( \mu l \) of 0.15 M cacodylate, pH 7.2, 3 mM CaCl\(_2\) (3 × 10 min at room temperature), warmed to 45 °C, mixed with 2% agar in 0.1 M cacodylate, pH 7.2, and pelleted. After rinsing in PBS (3 times for 10 min), the pellets were dehydrated in ethanol, embedded in Lowicryl white (SPI Supplies) and polymerized under UV light at 0 °C. Ultrathin sections were mounted on nickel grids that were floated on drops of 0.1% glycine in PBS for 10 min, then 1% BSA in PBS for 15 min, followed by antipiphopholipidase antiserum overnight at 4 °C (1:10 in 1% BSA/PBS). The sections were then stained with osmium tetroxide/uranyl acetate/lead citrate and examined in a CM10 transmission electron microscope.

Jurkat cells were grown in RPMI supplemented with 10% FCS at 37 °C in a humidified atmosphere of 95% air, 5% CO\(_2\). Cells (5 × 10\(^5\)) were incubated in RPMI, 1% FCS at 37 °C in medium supplemented with 10 mM Hepes. After incubation cells were pelleted by centrifugation (3,000 \( \times g \) for 5 min) and then washed (500 \( \mu l \) of STE), and the supernatant was centrifuged (13,000 \( \times g \) for 2 min) to give a mitochondria-enriched pellet and a cytosol- enriched supernatant. The mitochondrial pellet was washed in STE, and the supernatant was acetone-precipitated. Assays of the mitochondrial enzyme citrate synthase and of the cytosolic enzyme lactate dehydrogenase confirmed that this fractionation procedure was effective, with about 65% lactate dehydrogenase activity in the cytosol and about 35% citrate synthase activity in the mitochondrial fraction that contained about 40% of the total citrate synthase activity (36).

Jurkat cells were grown in RPMI supplemented with 10% FCS at 37 °C in a humidified atmosphere of 95% air, 5% CO\(_2\). Cells (5 × 10\(^5\)) were incubated in RPMI, 1% FCS at 37 °C in medium supplemented with 10 mM Hepes. After incubation cells were pelleted by centrifugation (3,000 \( \times g \) for 5 min) and washed in PBS, and the pellet was re-suspended in 2 ml of loading buffer. After incubation in 40 ml of loading buffer, cells were harvested from descending thoracic aortas and maintained in DMEM containing 1 g/l glucose and 10% FCS at 37 °C in a humidified atmosphere of 95% air, 5% CO\(_2\). Cells used in this study were between passages 5 and 10. For IBTP treatment 5 × 10\(^5\) cells were seeded in each well of 6-well plates in the growth medium and used when confluent. After incubation with IBTP the medium was removed, and the cells were suspended in loading buffer using a cell scraper. The toxicity of
IBTP was estimated from the amount of lactate dehydrogenase released after 24 h of incubation with human osteosarcoma 143B cells (37).

**RESULTS**

**Mitochondrial Localization of IBTP within Cells**—Human fibroblasts were incubated with IBTP (1 μM) for 1 h, and the intracellular localization of IBTP was then determined by immunocytochemistry using laser scanning confocal microscopy (Fig. 2A). IBTP binding co-localized with the mitochondrial enzyme cytochrome oxidase, and there was no IBTP binding outside mitochondria (Fig. 2A, upper panel). The uptake and binding of IBTP were prevented by dissipating the mitochondrial membrane potential with the uncoupler FCCP (Fig. 2A, lower panel). Both these results are consistent with but not conclusive for a mitochondrial localization of IBTP. As a further test for mitochondrial localization, immunogold electron microscopy was also employed in fibroblasts incubated with IBTP. The diffuse higher density structure characteristic of mitochondria imaged in cells using this technique is evident, with the black dots indicating the binding of the gold-labeled antibody surrounding these structures again consistent with a mitochondrial localization of IBTP within cells (Fig. 2B). The specificity of the antitriphenylphosphonium serum was confirmed by its detection of IBTP-labeled BSA on an immunoblot and by the selective blocking of this reaction by preincubation of the antiserum with TPMP (Fig. 3A). IBTP only showed signs of cytotoxicity at concentrations in excess of 25–50 μM (data not shown).

Those proteins labeled by incubating human fibroblasts with IBTP were separated by electrophoresis, transferred to nitrocellulose, and visualized by immunoblotting (Fig. 3B). There was extensive labeling of mitochondrial proteins within cells by IBTP (Fig. 3B, cell). That this labeling was due to the specific reaction of IBTP with mitochondrial proteins was confirmed by subfractionation of labeled cells into mitochondria- and cytosol-enriched fractions (Fig. 3B). This showed an identical pattern of labeling was prevented by abolishing the membrane potential by the membrane potential and labels matrix and mitochondrial proteins within cells and consequently can be used to probe their redox state. In the following series of studies the IBTP labeling of proteins in intact mitochondria was examined in more detail together with a proteomic analysis of the modifications involved.

**Location of IBTP-reactive Protein Thiols within Mitochondria**—Isolated mitochondria incubated with IBTP showed extensive labeling of mitochondrial protein thiols (Fig. 4A). Labeling was prevented by abolishing the membrane potential with the uncoupler FCCP (Fig. 4A), as would be expected if only thiols in the matrix and on the internal face of the inner membrane reacted with IBTP. This was confirmed by separating IBTP-treated mitochondria into mitoplast, outer membrane, and intermembrane space fractions; only the mitoplast fraction contained IBTP-labeled proteins, and the pattern of protein labeling was identical to that of intact mitochondria (data not shown). When IBTP-treated mitochondria were separated into membrane and matrix fractions, both fractions contained significant numbers of protein thiols, with more in the matrix fraction (Fig. 4B). The cohorts of thiol proteins labeled in the two fractions were clearly distinct, with several bands present in only the matrix or the membrane fraction (Fig. 4B). Therefore IBTP is accumulated into the mitochondria driven by the membrane potential and labels matrix and matrix-facing protein thiols which can then be detected by immunoblotting.

**Selectivity of IBTP Reaction with Mitochondrial Protein Thiols**—The thiol alkylating reagent NEM prevented the reaction of IBTP with mitochondrial thiols at a concentration that did not disrupt the membrane potential (Fig. 4A); therefore, its action was solely due to blocking mitochondrial thiols and not to preventing IBTP uptake. NEM also blocked the reaction of IBTP with BSA (data not shown) further indicating that the reaction of IBTP was predominantly with cysteine residues. The antiserum used was specific for IBTP-labeled proteins as its binding was blocked by preincubation with the simple triphenylphosphonium cation TPMP, and mitochondrial proteins that had not been treated with IBTP were not detected (Fig. 4B). The selectivity of protein thiol labeling could be modulated
for a further 15 min

A

at 30°C

lane was loaded with protein from 5

cell

detected by immunoblotting, and compared with intact cells ( ). Each

fractions. IBTP-labeled proteins were separated by SDS-PAGE,

and then pelleted and fractionated into mitochondria (mit) and cytosolic

cytosolic (cyt) fractions. IBTP-labeled proteins were separated by SDS-PAGE,

detected by immunoblotting, and compared with intact cells (cell). Each

lane was loaded with protein from 5 x 10^6 cells or a cell subfraction isolated from 2.5 to 5 x 10^6 cells. Increasing both the amount of protein in the cytosolic sample and the exposure time led to a pattern of labeling that was similar to that of the mitochondrial fraction (data not shown), presumably due to mitochondrial proteins released during cell homogenization.

C, effect of uncoupling on IBTP labeling in Jurkat cells. Jurkat cells were preincubated + FCCP (200 nM) for 10 min, incubated for a further 15 min + IBTP (5 μM), and then pelleted. IBTP-labeled proteins were separated by SDS-PAGE and detected by immunoblotting. Each lane was loaded with protein from 10^6 cells. D, effect of uncoupling on IBTP labeling in bovine aortic endothelial cells. Cells were grown to confluence in 6-well culture dishes and then incubated with the indicated IBTP concentrations + FCCP for 1 h, and then IBTP-labeled proteins were separated by SDS-PAGE and detected by immunoblotting.

by replacing the iodo functional group of IBTP with the less reactive bromo or chloro groups, and these compounds labeled mitochondrial thiols with the following reactivity order: IBTP > BrIBTP > ClIBTP (data not shown). To assess the potential of the higher intra-mitochondrial pH to enhance selective labeling of mitochondria, rates of reaction of IBTP with the free thiol of glutathione and with a protein thiol (BSA) were measured. The rates were found to be relatively slow (36 ± 7 x 10^-3 M^-1 s^-1 for glutathione and 53 ± 5 x 10^-3 M^-1 s^-1 for BSA at 37°C, pH 8) and were highly pH-sensitive, being 50–70% slower at pH 7.2 compared with pH 8. These data are consistent with a reaction occurring through nucleophilic displacement of iodide by a thiolate and supporting the role of matrix pH in enhancing the mitochondrial selectivity of IBTP labeling.

Limited Depletion of Mitochondrial Thiols by IBTP—Ideally a reagent for indicating thiol redox status would modify only a small pool of available protein thiol and have little effect on oxidative phosphorylation. Indeed, the relatively slow reaction between IBTP and thiols should label a small proportion of mitochondrial thiols without disrupting mitochondrial function by thiol depletion. This was supported by increased IBTP protein labeling at higher IBTP concentrations or longer incubation times (data not shown). Incubation with IBTP up to 10 μM did not deplete the mitochondrial glutathione pool to a significant extent, and even incubation with 10 μM IBTP for 30 min at 37°C depleted mitochondrial glutathione by less than 15% (data not shown). The effects of IBTP concentrations up to 25 μM on total mitochondrial protein thiols and on those free thiols exposed on non-denatured proteins were also not significant. We conclude that under conditions of low IBTP concentration, labeling of protein thiols occurs without significantly depleting overall mitochondrial thiol content.

Effect of IBTP on Mitochondrial Function—The effects of IBTP on phosphorylating or uncoupled respiration by isolated mitochondria were negligible below 50 μM (data not shown). There were small increases in coupled respiration at 25 μM IBTP, presumably due to slightly increased proton leak in the presence of the lipophilic cations (data not shown). Therefore, mitochondria can be incubated with up to 10–25 μM IBTP without significant effects on oxidative phosphorylation as would be expected if a small subset of the available thiols in functionally important proteins was being modified by the re-
Mitochondrial Thiol Proteins

IBTP Labeling of Respiratory Complexes Identified by Blue Native Gel Electrophoresis—A drawback with the use of conventional two-dimensional electrophoresis is that many hydrophobic mitochondrial inner membrane proteins cannot be separated because they precipitate in the isoelectric focusing gel (38). This fraction contains many polypeptides of interest in the respiratory complexes that are thought to contain thiols susceptible to modification by reactive oxygen and nitrogen species. An alternative approach is to use blue native gel electrophoresis (BN-PAGE) in which respiratory complexes are separated in an intact and native form (27). Mitochondria were incubated with IBTP, and respiratory complexes were then separated by BN-PAGE, transferred to nitrocellulose, and probed with antitriphenylphosphonium antiserum (Fig. 6).

Prominent among the IBTP-labeled protein complexes was one the size of Complex I (~900 kDa), and its identity was confirmed by probing a parallel sample with a Complex I-specific antibody (Fig. 6A). Other protein complexes were labeled by IBTP, but their intensity was less than for Complex I as indicated by the overexposed Complex I band (Fig. 6B). The molecular weights of these IBTP-reactive bands were similar to Complex II (succinate dehydrogenase; 130 kDa) and Complex IV (cytochrome oxidase; 200 kDa), and these identities were confirmed by probing parallel samples with specific antibodies (Fig. 6, C and D).

The large mitochondrial complexes initially isolated by BN-PAGE could be resolved as individual polypeptides by carrying out SDS-PAGE in the second dimension (Fig. 6E). This was done for identical samples from IBTP-labeled mitochondria, and the resulting gels were then either stained with Coomassie Blue or transferred to nitrocellulose and probed with antitriphenylphosphonium antiserum (Fig. 6F). A number of IBTP-labeled polypeptides were seen, including polypeptides from all the protein complexes detected by immunoblotting of the BN-PAGE. In addition many more IBTP-labeled polypeptides were evident after the second dimension SDS-PAGE than would be predicted from immunoblots of the BN-PAGE. This increased sensitivity following second dimension SDS-PAGE is probably due to removal of the high background found for BN-PAGE immunoblots that limit sensitivity and to greater binding of the antiserum to the denatured proteins.

Loss of Mitochondrial Protein Thiols in Response to Oxidative Stress—To determine how protein thiols change redox state in response to oxidative stress, we measured the effect of oxidants on IBTP binding to mitochondrial protein thiols (Fig. 7). Addition of diamide, a thiol-selective oxidant, led to a significant decrease in labeling of mitochondrial proteins by IBTP (Fig. 7A). To eliminate the possibility that decreased IBTP labeling merely reflected less IBTP uptake due to disruption of the membrane potential, the effect of diamide on membrane potential was shown to be negligible (Fig. 7A, inset panel).

Addition of tert-butyl hydroperoxide (tBHP) also decreased labeling of mitochondrial thiols by IBTP without disrupting the membrane potential (Fig. 7B). This occurs because tBHP is a glutathione peroxidase substrate that oxidizes mitochondrial glutathione to glutathione disulfide that then forms protein mixed disulfides (39), and also as a consequence of oxidative stress induced by the peroxide itself.

To investigate the interaction of reactive nitrogen species with mitochondrial thiols, we studied the effects of peroxynitrite and SNAP on IBTP labeling of mitochondrial proteins (Fig. 7, C and D). Peroxynitrite contributes to oxidative stress in vivo and is more reactive than its precursors, superoxide and

---

**Figure 5. Resolution of IBTP-labeled mitochondrial proteins by two-dimensional electrophoresis.** Mitochondria, matrix, or membrane fractions (200 μg of protein) from IBTP-treated mitochondria were separated by two-dimensional gel electrophoresis. Identical gels were either Coomassie-stained or transferred to nitrocellulose and probed with antitriphenylphosphonium antiserum. A, mitochondria, IEF pH 3–10. B, mitochondria, IEF pH 4–7. C, membrane, IEF pH 3–10. D, matrix, IEF pH 4–7. Control immunoblots of mitochondria that had not been reacted with IBTP gave no immunoreactivity (data not shown). Circles show spots present in both Coomassie- and IBTP-stained gels, and squares show spots present only in the Coomassie-stained gels.

---

The figure shows a two-dimensional gel electrophoresis result with three panels (A, B, and C) and a diagram with labeled axes. The gel electrophoresis panels show Coomassie-stained gels and IBTP-labeled gels with corresponding protein bands. The diagram includes a scale for molecular weight and pH range.
Mitochondrial Thiol Proteins

Fig. 6. Reaction of IBTP with oxidative phosphorylation complexes. Mitochondria (1 mg of protein/ml) were incubated for 5 min at 30 °C in KCl medium supplemented with 10 mM glutamate/malate and IBTP (25 μM). The mitochondria were pelleted, and 250 μg of protein was separated by blue native PAGE, transferred to nitrocellulose, and probed with antitriphenylphosphonium antiserum, or with monoclonal antibodies against respiratory complexes. Where indicated FCCP (200 nM) was present or IBTP was omitted. A, Complex I. Parallel blots were probed with antiserum against triphenylphosphonium and with a monoclonal antibody against succinate dehydrogenase. Parallel blots were probed with antiserum against triphenylphosphonium and with a monoclonal antibody against cytochrome oxidase (COX). B, respiratory complexes. The blot was probed with antitriphenylphosphonium antiserum and was overexposed to visualize further IBTP-labeled bands in addition to Complex I (overexposed band). C, cytochrome oxidase. Parallel blots were probed with antiserum against triphenylphosphonium and with a monoclonal antibody against cytochrome oxidase (COX). D, succinate dehydrogenase. Parallel blots were probed with antiserum against triphenylphosphonium and with a monoclonal antibody against succinate dehydrogenase (SDH). Data are typical of results repeated on several gels for each sample. E, two-dimensional separation of mitochondrial proteins. Mitochondrial proteins were separated by BN-PAGE in the first dimension followed by SDS-PAGE in the second dimension. Identical gels were then either stained with Coomassie Blue or probed with antitriphenylphosphonium antiserum. The approximate locations of mitochondrial oxidative phosphorylation complexes after BN-PAGE are indicated.

DISCUSSION

The importance of redox changes to mitochondrial thiol proteins during oxidative stress and cell death is well recognized, but direct measurement of the modification of different mitochondrial protein thiols by oxidative stress has not been undertaken. By using the novel compound IBTP, specific labeling of mitochondrial protein thiols was demonstrated in both intact cells and the isolated organelle. From the pattern of IBTP labeling on immunoblots, it was clear that mitochondria contain a number of relatively abundant proteins with IBTP-reactive thiols. Most of these proteins were in the matrix, but a significant proportion of membrane proteins also contained reactive thiols on their matrix-facing surfaces. In future studies, IBTP labeling will enable individual mitochondrial thiol proteins to be isolated and identified by spot excision from the gel followed by N-terminal sequencing or in-gel trypsin digestion in conjunction with mass spectrometry. The mass shift due to the butyltriphenylphosphonium adduct (+316.1381 for the singly charged peptide) will also facilitate identification by mass spectrometry of tryptic peptides containing active thiol residues.

Changes in thiol redox state prevented IBTP labeling of protein thiols, facilitating their isolation by electrophoresis and immunoblotting. The reactivity of the majority of mitochondrial thiol proteins with IBTP decreased on oxidizing the glutathione pool with diamide (D) (Fig. 7D). Therefore, loss of IBTP labeling can be used to measure redox changes in mitochondrial thiols in response to oxidative stress. From these studies we were also able to conclude that oxidation of the mitochondrial glutathione pool or the formation of ROS and RNS has a dramatic effect on the redox state of mitochondrial thiol proteins.

Treatment with diamide, a reagent that reacts with both protein and low molecular weight thiols, decreased the overall intensity of protein labeling by IBTP (Fig. 7A). This could be due to a generalized decrease in the IBTP reactivity of all thiol proteins, to the selective loss of particular protein thiols, or to a combination of both processes. To investigate this, we compared the proteins labeled by IBTP in diamide-treated and control mitochondria using two-dimensional immunoblots (Fig. 7E). Immunoblots of diamide-treated samples were developed until their intensities were similar to controls to facilitate comparison of the patterns of thiol protein labeling. The pattern of IBTP labeling seen on two-dimensional immunoblots was largely unchanged by treatment with diamide, suggesting that most mitochondrial protein thiols are susceptible to oxidative stress to a similar degree with this reagent. Therefore the decrease in IBTP labeling caused by diamide seen in Fig. 7A is due to the formation of mixed disulfides by most exposed protein thiols. However, some protein thiols were particularly susceptible to alteration by oxidative stress as IBTP labeling was lost following treatment with diamide (arrows, Fig. 7E). A further point of interest was that some mitochondrial thiol proteins increased their IBTP reactivity on treatment with diamide, tBHP, SNAP, and NEM (arrows, Fig. 7, A, B, and D, and Fig. 4A). Therefore, in response to oxidative stress most mitochondrial protein thiols are affected to a similar extent; in addition there is a subset of protein thiols that are particularly susceptible to changes in redox state. In all cases changes in the reactivity of protein thiols with IBTP can be used to identify those proteins that change their thiol redox state in response to oxidative stress.

Changes in thiol redox state prevented IBTP labeling of protein thiols, facilitating their isolation by electrophoresis and immunoblotting. The reactivity of the majority of mitochondrial thiol proteins with IBTP decreased on oxidizing the glutathione pool with diamide or tBHP (39). As expected, exposure to the S-nitrosothiol SNAP or to peroxynitrite did not have as extensive effects on the redox state of mitochondrial thiol proteins as direct oxidation of the glutathione pool. These changes are likely to occur due to S-nitrosation of proteins or as a...
Mitochondrial Thiol Proteins

Secondary consequence of oxidation or $S$-nitrosation of mitochondrial glutathione. Very little is known about intraganelle signaling in the mitochondrion. By analogy with established pathways in the cytosol, one can propose that thiol modification of key metabolic or signaling molecules occurs within the organelle due to the formation of protein mixed disulfides or $S$-nitrosated species. Interestingly, oxidant production in the cytosol could coordinate responses in the mitochondrion through thiol modification, in addition to activating signaling pathways in the cytosol. This is supported by the finding that SNAP modified intramitochondrial protein thiols when added to intact mitochondria. The protein-glutathione mixed disulfides formed will be regenerated after oxidative stress by thioredoxin/thioredoxin reductase, glutaredoxin/glutaredoxin reductase, or by re-equilibration with glutathione. It is anticipated that reductive processes involving GSH will determine the persistence and stability of modified thiol proteins. Indeed in a metabolic regulatory role GSH is likely to be critical in controlling the duration of a signal in an analogous fashion to a phosphatase.

Analysis of the mitochondrial inner membrane fraction, which contains key components in electron transport and apoptosis, was particularly interesting. Using IBTP labeling in conjunction with BN-PAGE enabled us to confirm the presence of free thiol residues on the matrix surface of respiratory Complexes I, II, and IV. Whereas the presence of reactive thiols on these complexes is known, their locations and roles are uncertain; IBTP labeling will help address these points (1, 42–45). Using a combination of proteomics approaches, we have uncovered a number of candidate protein thiols that are particularly susceptible to thiol modification, and these are now being investigated as potential regulators or redox sensors in the response of mitochondria to oxidative stress.

Interestingly, some protein thiols increased their reactivity with IBTP on exposure to diamide, SNAP, or NEM. Whereas the cause of this unexpected finding is uncertain, there are a
number of possibilities. Oxidative stress may cause conformational changes that render some protein thiols more reactive, either by increasing their exposure to the matrix or by decreasing their pKaq values by moving them relative to charged residues. Alternatively, the increased thiol reactivity may follow alteration to an iron-sulfur center that exposes a cysteine that had been blocked previously by an iron atom.

To conclude, we have developed a procedure to label mitochondrial thiol proteins within living cells that enables us to follow changes in their redox state. By using this procedure we have shown that there are a number of active thiols on the surface of mitochondrial matrix and membrane proteins that change their redox state in response to both glutathione oxidation and exposure to S-nitrosating agents or peroxynitrite. Among these are individual protein thiols that are particularly responsive to oxidative stress, either decreasing or increasing their IBTP binding. The development of a selective mitochondrial thiol reagent enables the technology of proteomics to be focused on this important subset of mitochondrial proteins, and will help characterize those mitochondrial thiol proteins that change redox state during oxidative stress, apoptosis, and aging.

REFERENCES

Specific Modification of Mitochondrial Protein Thiols in Response to Oxidative Stress: A PROTEOMICS APPROACH
Tsu-Kung Lin, Gillian Hughes, Aleksandra Muratovska, Frances H. Blaikie, Paul S. Brookes, Victor Darley-Usmar, Robin A. J. Smith and Michael P. Murphy

doi: 10.1074/jbc.M110797200 originally published online February 22, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M110797200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 43 references, 14 of which can be accessed free at http://www.jbc.org/content/277/19/17048.full.html#ref-list-1