Published in final edited form as:

Methods Mol Biol. 2019 ; 1967: 211–227. doi:10.1007/978-1-4939-9187-7_13.

Interrogation of Functional Mitochondrial Cysteine Residues by Quantitative Mass Spectrometry.

Daniel W. Bak, Eranthie Weerapana

Department of Chemistry, Boston College, Chestnut Hill, MA

Summary:

Mitochondria are cellular sites of diverse redox biology, including ROS production, iron-sulfur biogenesis, and secondary metabolism, which all rely on proteogenic reactive cysteine residues. Mass spectrometry-based proteomic methods to monitor the reactivity and functionality of cysteine residues across complex proteomes have greatly expanded over the past decade. Here we describe a mitochondrial isolation procedure coupled with cysteine-reactive IA-labeling that affords identification and characterization of functional mitochondrial cysteine residues that were heretofore inaccessible to whole cell proteomic analysis.

Keywords

Mitochondria; Cysteine Reactivity; Mass Spectrometry; Proteomics; Activity-based Proteomic Profiling (ABPP)

1. Introduction

Mitochondria are eukaryotic subcellular organelles that regulate oxidative metabolism and mediate cellular redox homeostasis. Approximately 1,500 proteins are estimated to associate with the mitochondria under various cellular conditions, and constitute the human mitochondrial proteome¹. Mitochondrial proteins are exposed to large fluxes in the surrounding redox environment due to (patho)physiological disruptions in mitochondrial redox homeostasis². Reactive oxygen and nitrogen species (ROS/RNS) that are generated during normal oxidative metabolism as well as under certain pathological conditions can dynamically regulate mitochondrial proteins through oxidative post-translational modifications (PTMs)^{3–7}. These PTMs, which affect protein localization^{8,9}, catalytic activity^{10,11}, and protein-protein interactions¹², primarily occur on cysteine residues. The various cysteine oxidative PTMs include oxidation to sulfenic/sulfinic/sulfonic acids, S-nitrosation, S-glutathionylation, and disulfide-bond formation^{13,14}. For example, oxidation of a cysteine residue on uncoupling protein 1 (UCP1)¹⁵ and S-nitrosation of a cysteine residue on complex I¹⁶, both affect protein function. These oxidative modifications result in a loss in cysteine reactivity, which can be monitored in global cysteine-profiling studies using cysteine-reactive chemical probes and quantitative mass spectrometry (MS).

Coupling of cysteine-reactive chemical probes, such as an iodoacetamide-alkyne (IA), with quantitative mass spectrometry (MS) has enabled the global interrogation of the cysteine proteome to identify functional cysteines and sites of PTMs^{3–7}, referred to as isoTOP-ABPP. Global cysteine-profiling studies typically involve MS analysis of unfractionated cell lysates^{3–6} or more recently live cells in culture^{7,17}, which often provide limited coverage of the mitochondrial proteome. Poor mitochondrial coverage is likely the result of low mitochondrial protein abundance relative to highly abundant cytosolic proteins, resulting in suppression of mitochondrial peptide MS signals. In recent global proteomic studies, improved coverage of the mitochondrial proteome was achieved through selective enrichment of mitochondrial proteins prior to MS-based analysis, by enrichment and isolation of purified mitochondria¹⁸ (Figure 1A). We recently reported a variation on the standard isoTOP-ABPP platform, in which purified intact mitochondria were labeled with IA-alkyne, instead of whole cell lysates¹⁹ (Figure 1). By labeling intact mitochondria, we achieve both increased labeling of mitochondrial cysteine residues and preserve redox sensitive oxidative cysteine PTMs. We have successfully applied this platform to measure the reactivity of > 500 mitochondrial cysteines and identify novel mitochondrial sites of GSNO-mediated S-nitrosation. This platform has also been successfully applied to compare changes in mitochondrial cysteine reactivity between two isogenic cell lines (data unpublished). Here we provide a detailed protocol for the applying this platform to quantitatively monitor changes in cysteine reactivity between two biological samples.

The mitochondrial cysteine enrichment protocol begins with the isolation of “pure” intact mitochondria from two cultured cell samples (Figure 1A), followed by a quantitative cysteine enrichment platform to measure changes in cysteine reactivity between cell lines (Figure 1B and C). Examples of possible experimental studies include comparing a control cell line (Proteome A) to an isogenic knock-out or an oxidant treated cell sample (Proteome B). Whole cells are gently homogenized to disrupt the cell membrane (Step 1), and a “crude” mitochondrial fraction is isolated by differential centrifugation (Step 2). Subsequently, a “pure” mitochondrial fraction is isolated by gradient centrifugation using a step-wise percoll gradient (Step 3). Intact mitochondria are then labeled with the IA-alkyne probe (Step 4) to preserve cysteine modification and/or oxidation state. The mitochondria are quickly lysed and conjugated to either Azo-L (Proteome A) or Azo-H (Proteome B) tags through copper-catalyzed azide-alkyne cycloaddition, CuAAC (Step 5). The two proteomes are mixed together and subjected to enrichment on streptavidin beads (Step 6), on-bead tryptic digestion (Step 7), and cleavage of the azobenzene linker by sodium hydrosulfite (Step 8). Isotopically labeled peptides are then analyzed by LC/LC-MS/MS on a high-resolution Orbitrap Mass Spectrometer (Thermo Scientific), using a multidimensional protein identification technology (MudPIT) method (Step 9). IA-labeled peptides are identified using SEQUEST, and light:heavy ratios (R) are quantified by CIMAGE to provide a measure of relative abundance of a peptide between the two proteome samples (Step 10). A typical experiment can identify over 1,500 mitochondrial cysteine residues, and quantify changes in their reactivity upon oxidative stress conditions, including treatment with exogenous oxidants or isogenic knock-out.

2. Materials

2.1. Mitochondrial isolation components

1. Mitochondrial Isolation Buffer (MIB): 10 mM Tris/MOPS, 1 mM EDTA/Tris, 200 mM Sucrose, pH 7.4 (*see* Note 1)
2. 0.1 M Tris/MOPS (100 mM Tris in distilled water, adjusted to pH 7.4 with MOPS powder) (Sigma-Aldrich, St. Louis, MO, USA)
3. of 0.1 M EDTA/Tris (100 mM EDTA in distilled water, adjusted to pH 7.4 with Tris powder) (Sigma-Aldrich, St. Louis, MO, USA)
4. 1 M sucrose (1 M sucrose in distilled water) (Affymetrix, Santa Clara, CA, USA)
5. 100% percoll solution (23% colloidal suspension in water) (Sigma-Aldrich, St. Louis, MO, USA) (*see* Note 2)
6. 80% percoll solution: 4 parts 100% percoll, 1 part MIB (5×)
7. 52% percoll solution: 80% percoll solution diluted with $0.54 \times$ volumes of MIB
8. 26% percoll solution: 80% percoll solution diluted with $2.07 \times$ volumes of MIB
9. PBS, Phosphate Buffered Saline (Corning, Corning, NY, USA)

2.2. Western blotting components

1. 10X Loading buffer (Invitrogen, Carlsbad, CA, USA)
2. SDS-PAGE gel (BioRad, Hercules, CA, USA)
3. Precision plus protein all blue standards (BioRad, Hercules, CA, USA)
4. SDS-PAGE running buffer: 3 g tris-base (BioRad, Hercules, CA, USA), 14.4 g glycine (BioRad, Hercules, CA, USA), 1 g SDS (BioRad, Hercules, CA, USA) in 1 L water.
5. Nitrocellulose membranes (GE Healthcare Life Sciences, Pittsburgh, PA, USA)
6. Transfer buffer: 3 g tris-base (BioRad, Hercules, CA, USA), 14.4 g glycine (BioRad, Hercules, CA, USA) in 1 L of methanol:water 1:4.
7. Tris-buffered saline (TBS): 6 g tris-base (BioRad, Hercules, CA, USA), 11.7 g NaCl (Fisher Scientific, Pittsburgh, PA, USA), pH 7.4 in 1 L water.
8. TBS containing 0.05% Tween-20 (TBST)
9. Blocking solution (5% milk in TBST)
10. Rabbit anti-GAPDH antibody: 1:2,000 in blocking solution (Cell Signaling, Danvers, MA, USA). Store at 4 °C.
11. Rabbit anti-calreticulin antibody: 1:1,000 in blocking solution (Cell Signaling, Danvers, MA, USA). Store at 4 °C.
12. Mouse anti-complex V inhibitor protein antibody: 1:1,000 in blocking solution (Cell Signaling, Danvers, MA, USA). Store at 4 °C.

13. Anti-rabbit IgG HRP-linked antibody: 1:2,000 in blocking solution (Cell Signaling, Danvers, MA, USA). Store at 4 °C.
14. Anti-mouse IgG HRP-linked antibody: 1:2,000 in blocking solution (Cell Signaling, Danvers, MA, USA). Store at 4 °C.
15. SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, Rockford, IL, USA)

2.3. Proteome preparation, IA-alkyne labeling, and CuAAC components

1. Bio-Rad DC protein assay kit (BioRad, Hercules, CA, USA)
2. IA-alkyne probe (10 mM solution in DMSO)²⁰ (*see Note 3*)
3. Tris 2-carboxyethyl phosphine (TCEP) (50 mM solution in water) (Sigma-Aldrich, St. Louis, MO, USA) (*see Note 4*)
4. Tris[(1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl]amine (“ligand”) (1.7 mM solution in DMSO:t-butanol 1:4) (Sigma-Aldrich, St. Louis, MO, USA)
5. Azo-L/Azo-H tags (5 mM solutions in DMSO)²¹ (*see Note 3*)
6. Copper (II) sulfate (50 mM solution in water) (Sigma-Aldrich, St. Louis, MO, USA)
7. Methanol (Fisher Scientific, Pittsburgh, PA, USA)
8. Sodium dodecyl sulfate (SDS) (0.2% and 1.2% solutions in PBS) (BioRad, Hercules, CA, USA)

2.4. Streptavidin enrichment, on-bead trypsin digestion, and sodium-hydrosulfite cleavage components

1. Streptavidin agarose beads (Thermo Scientific, Rockford, IL, USA)
2. Urea (2 M and 6 M solutions in PBS) (Sigma-Aldrich, St. Louis, MO, USA) (*see Note 4*)
3. Screw-Cap Centrifuge Tubes (Thomas Scientific, Swedesboro, NJ, USA)
4. Dithiothreitol (DTT) (200 mM solution in water) (Invitrogen, Carlsbad, CA, USA) (*see Note 4*)
5. Iodoacetamide (400 mM solution in water) (Acros Organics, NJ, USA) (*see Note 4*)
6. Calcium chloride (100 mM solution in water) (Sigma-Aldrich, St. Louis, MO, USA)
7. Trypsin solution: 20 µg of sequencing-grade trypsin reconstituted in 40 µL of trypsin buffer (provided in package) (Promega, Madison, WI, USA) (*see Note 5*)
8. Sodium hydrosulfite (50 mM solution in PBS) (Sigma-Aldrich, St. Louis, MO, USA) (*see Note 4*)

9. Formic acid (Fisher Scientific, Torrance, CA, USA) (*see* Note 6)

2.5. Mass spectrometry analysis

1. Fused-silica capillary tubing (100 and 250 μ m internal diameter) (Agilent, Santa Clara, CA, USA)
2. Aqua C18 reverse-phase resin (Phenomenex, Torrance, CA, USA)
3. Partisphere strong cation exchange (SCX) resin (Phenomenex, Torrance, CA, USA)
4. PC77 Pressure Injection Cell (Next Advance, Troy, NY, USA)
5. Model P-2000 Tip Puller (Sutter Instruments, Novato, CA, USA)
6. Buffer A: 95% water, 5% acetonitrile, 0.1% formic acid (*see* Note 6)
7. Buffer B: 20% water, 80% acetonitrile, 0.1% formic acid (*see* Note 6)
8. Buffer C: 95% water, 5% acetonitrile, 0.1% formic acid, 500 mM ammonium acetate (*see* Note 6)
9. Water, LC/MS Grade (Fisher Scientific, Pittsburgh, PA, USA)
10. Acetonitrile, LC/MS Grade (Fisher Scientific, Pittsburgh, PA, USA)
11. Formic Acid, LC/MS Grade (Fisher Scientific, Pittsburgh, PA, USA)
12. Ammonium acetate, LC/MS Grade (Fisher Scientific, Pittsburgh, PA, USA)
13. Orbitrap LTQ Discovery (Thermo Scientific, Rockford, IL, USA)
14. Agilent 1200 Series HPLC (Agilent, Santa Clara, CA, USA)

3. Methods

3.1. Mitochondrial isolation (“Crude”)²²

1. Resuspend cell pellets from Proteome A and Proteome B in 1 volume of mitochondrial isolation buffer (MIB) (*see* Note 7), then centrifuge at 1,000 *g* for 5 min. Repeat wash in MIB two more times.
2. Resuspend cell pellets in 10 volumes of MIB and homogenize with 30 strokes (3 $\times$ 10 strokes, rest on ice between) with a Teflon pestle in a pre-chilled glass potter. (*see* Note 8)
3. Centrifuge homogenate at 600 *g* for 10 minutes at 4 °C. Collect supernatant and discard pellet. Centrifuge again at 600 *g* for 10 minutes at 4 °C, and save supernatant. (*see* Note 9)
4. Centrifuge supernatant at 12,500 *g* for 10 minutes at 4 °C. Discard supernatant, resuspend pellet in ~5 volumes of MIB, and centrifuge again at 12,500 *g* for 10 minutes at 4 °C, saving the pellet. (*see* Note 10)
5. This final pellet is a “crude” mitochondrial preparation. (*see* Note 11)

3.2. Mitochondrial isolation (“Pure”)¹⁸

1. Prepare a step-wise percoll density gradient in a 3-mL ultracentrifuge tube. The gradient consists of 0.5 mL of 80% percoll solution, 1 mL of 52% percoll solution, and 1 mL of 26% percoll solution. (*see Note 12*)
2. Resuspend “crude” mitochondrial pellets in ~1 volume of MIB. Gently overlay the “crude” mitochondrial sample onto the gradient and centrifuge at 21,500 *g* for 45 minutes at 4 °C. (*see Note 13*)
3. Using a 16-gauge needle, extract the white mitochondrial band from the 52:26% percoll interface. (*see Note 14*)
4. Add MIB to bring the volume up to ~2 mL and centrifuge at 12,500 *g* for 10 minutes at 4 °C. Discard the supernatant, resuspend the pellet in ~5 volumes of MIB, and centrifuge again at 12,500 *g* for 10 minutes at 4 °C.
5. This final pellet is a “pure” mitochondrial preparation.

3.3. Proteome preparation

1. Wash mitochondrial pellets from Proteome A and Proteome B three times in PBS, centrifuging at 12,500 *g* for 10 minutes at 4 °C after each wash. (*see Note 15*)
2. Gently resuspend mitochondrial pellets in 0.5 mL of PBS, and measure protein concentrations by Bio-Rad DC protein assay kit according to manufacturer’s instructions. (*see Note 16*)
3. Dilute intact mitochondrial solutions to 1 mg/mL with PBS. (*see Note 17*)

3.4. Western blotting

1. For both proteomes, combine 20 µL of 1 mg/mL intact “pure” mitochondria (20 µg) with 2 µL of 10x loading buffer and vortex to mix. Repeat with equal amount of reserved “crude” mitochondria, and whole cell lysates as a control. For all three samples load entire volume into a separate well of a 12.5 % SDS-PAGE gel and load 20 µL of precision plus protein standards on either side of the three wells.
2. Run gel at 150 V until dye front reaches the bottom of the gel.
3. Transfer to nitrocellulose membrane at 80 V for 60 minutes. Using the protein standard lanes as a guide, carefully cut the nitrocellulose membrane into uneven thirds, so that the bottom, center, and top parts of the membrane contain the 12.5 kDa Complex V inhibitor protein, the 36 kDa GAPDH protein, and the 48 kDa Calreticulin protein, respectively. (*see Note 18*)
4. Incubate membranes shaking in blocking solution overnight at 25 °C, then incubate with appropriate primary antibody (anti-complex V inhibitor protein, anti-GAPDH, and anti-calreticulin) for 3 hours at 25 °C. Wash membranes three times with blocking solution, with 5-minute incubation each time.

5. Incubate membrane shaking with secondary antibody (anti-mouse IgG: complex V inhibitor protein, anti-rabbit IgG: GAPDH and calreticulin). Wash membrane three times with TBST and three times with TBS, with 5-minute incubation each time.
6. Visualize blot with SuperSignal West Pico Chemiluminescent Substrate according to manufacturer's instructions.

3.5. IA-labeling and CuAAC

1. Add 500 μ L of intact mitochondria in PBS (1 mg/mL) to two 1.5 mL Eppendorf tubes for both Proteome A and Proteome B (4 tubes total). To each tube add 5 μ L of 10 mM IA-alkyne (100 μ M final concentration). Mix thoroughly by inversion and allow to incubate at room temperature (RT) for 1 hour. (*see* Note 19)
2. Lyse each of the four tubes of intact mitochondria by sonication for 30 seconds at 4 °C.
3. Add 10 μ L of either 5 mM Azo-L (Proteome A) or Azo-H (Proteome B), 20 μ L of fresh 50 mM TCEP solution (*see* Note 4), 30 μ L of 1.7 mM "ligand" solution, and 10 μ L of 50 mM copper (II) sulfate solution to each of the four tubes and vortex. Allow reaction to incubate at RT for 1 hour, vortexing to resuspend precipitating protein every 15 minutes. The proteins will precipitate at this stage and the solution will turn cloudy.
4. After 1 hour incubation, combine tubes pairwise (one tube of Proteome A with one tube of Proteome B), and centrifuge at 6,500 *g* for 5 minutes. Discard supernatant and resuspend pellet in 500 μ L ice-cold methanol by brief sonication. Combine remaining two tubes together, and centrifuge at 6,500 *g* for 5 minutes at 4 °C. Wash final pellet again in 500 μ L of ice-cold methanol.

3.6. Streptavidin enrichment, on-bead trypsin digestion, and sodium-hydrosulfite cleavage

1. Resuspend pellet in 1 mL of 1.2% SDS in PBS, sonicating to completely dissolve protein pellet, and heat at 85–90 °C for 10 minutes. Centrifuge at 6,500 *g* for 5 minutes at RT to pellet copper, and transfer supernatant to 15 mL conical tube with 5 mL PBS (0.2% final concentration of SDS).
2. Aliquot 200 μ L of streptavidin agarose slurry (~100 μ L of beads) into a 1.5 mL Eppendorf and wash three times with 500 μ L of PBS. Transfer washed beads to 15 mL conical tube with sample and incubate overnight rotating at 4 °C.
3. After overnight incubation at 4 °C, incubate samples with rotation at RT for 2 hours. Centrifuge samples at 1,400 *g* for 5 minutes, decant supernatant, add 5 mL of 0.2% SDS in PBS and incubate rotating for 10 minutes at RT.
4. Centrifuge sample at 1,400 *g* for 5 minutes, decant supernatant and wash beads three times with 5 mL PBS followed by three washes with 5 mL water. (*see* Note 20)

5. Transfer beads to screw-cap Eppendorf tubes with three 167 μL aliquots of 6 M Urea in PBS (500 μL total volume). Add 25 μL of fresh 200 mM DTT (*see Note 4*) and incubate samples at 65 °C for 20 minutes, mixing by inversion after 10 minutes. Add 25 μL of fresh 400 mM iodoacetamide after tube cools (*see Note 4*) and incubate with rotation at 37 °C for 30 minutes.
6. Add 950 μL of PBS to sample (2M Urea final concentration), centrifuge at 1,400 g for 5 minutes at RT, and remove supernatant. Add pre-mixed solution of 200 μL of 2 M Urea in PBS, 2 μL of 100 mM calcium chloride, and 4 μL of trypsin solution (*see Note 5*). Incubate reaction overnight rotating at 37 °C.
7. After overnight incubation remove supernatant, and wash beads three times with 500 μL of PBS followed by three washes with 500 μL of water. (*see Note 21*)
8. Add 50 μL of freshly made 50 mM sodium hydrosulfite solution to the beads (*see Note 4*), incubate rotating at RT for one hour, centrifuge at 1,400 g for 5 minutes, and then collect supernatant. Repeat this step twice more, using 75 μL of 50 mM sodium hydrosulfite solution, and combining collected supernatants with first fraction. (*see Note 22*)
9. Wash beads twice with 75 μL of water and combine collected washes with previous fractions, for a total volume of 350 μL . Add 17.5 μL of formic acid to sample, vortex, and store at –20 °C until mass spectrometry analysis.

3.7. Mass spectrometry analysis using MudPIT

1. Using a pressure injection cell, load a 5 μm tip with 10 cm of Aqua C18 reverse-phase resin, followed by 4 cm of strong cation exchange (SCX) resin and pressure load a desalting column with 4 cm of Aqua C18 reverse-phase resin. (*see Note 23*)
2. Equilibrate both the tip and desalting column by pressure loading Buffer B for 10 minutes, followed by Buffer A for 20 minutes. Thaw sample and pressure load entire sample onto desalting column.
3. Assemble tip and desalting column together and position on a nanospray stage attached to an Orbitrap LTQ Discovery mass spectrometer equipped with an Agilent 1200 series HPLC.
4. Run 5-step MudPIT elution program (Table 1), with a flow rate of –0.25 $\mu\text{L}/\text{min}$ and a spray voltage set to 2.75 kV. A full MS scan (FTMS; 400–1800 MW) is followed by 8 data-dependent scans (ITMS) of the n th most intense ions with dynamic exclusion enabled (repeat count =1; exclusion size list = 300, exclusion duration = 30s)

3.8. Data analysis

1. The tandem MS data is searched against a human reverse-concatenated nonredundant FASTA database containing uniprot identifiers using the SEQUEST algorithm for peptide identification²³.

2. Static modifications of +57.0215 m/z (iodoacetamide alkylation) and differential modifications of +456.2849 (Azo-L) or +462.2987 (Azo-H) on cysteine are specified to account for the presence of probe modification
3. SEQUEST output files were filtered using DTASelect 2.0²⁴, applying the -trypstat and -modstat options. Peptides are allowed from a single locus (-p 1), but must be fully tryptic (-y 2), with desired modification (-m 0), and a deltaCN score greater than 0.06 (-d 0.06).
4. Quantification of light/heavy (Azo-L/Azo-H) ratios (R) is performed using the CIMAGE quantification package⁵.
5. Annotation of cysteine residues as mitochondrial is achieved by searching protein IDs against the databases, including the mitoCarta²⁵, found on the MitoMiner website (<http://mitominer.mrc-mbu.cam.ac.uk>)²⁶.

4. Notes

1. Combine 10 mL of 0.1 M Tris/MOPS (100 mM Tris in distilled water, adjusted to pH 7.4 with MOPS powder), 1 mL of 0.1 M EDTA/Tris (100 mM EDTA in distilled water, adjusted to pH 7.4 with Tris powder), and 20 mL of 1 M sucrose (1 M sucrose in distilled water). Adjust volume to 100 mL with distilled water and adjust pH to 7.4. MIB is a standard buffer used to isolate mitochondria from cell culture, and has previously been found to successfully isolate mitochondria from MEFs in culture²². Other isolation buffers, such as those which contain mannitol instead of sucrose could also be utilized here.
2. Percoll is supplied as a 23% colloidal suspension in water. This is considered the 100% percoll solution. The solutions of 80, 52, and 26% percoll in MIB are 18.5, 12, and 6% colloidal suspensions, respectively.
3. IA-alkyne probe²⁰ and Azo-L/Azo-H tags²¹ are made in house and stored as 10x stocks in DMSO at -20 °C.
4. TCEP, urea, DTT, iodoacetamide, and sodium hydrosulfite solutions need to be made fresh immediately prior to use.
5. Trypsin solution should be made fresh immediately prior to use and can be stored at 4 °C for 1 week.
6. Mass Spectrometry Grade Reagents should be used.
7. In order to get enough material for proteomic experiments, we have found that 5–10 plates (20 cm) of fully confluent cells are required. This is highly dependent on the cell line and will need to be determined by the researcher. Mitochondria should be extracted from these pellets the same day they are harvested, as freeze thawing could damage cell structure and mitochondria.
8. The teflon pestle, glass potter combination represents a gentle cell lysis platform, which preserves mitochondrial integrity. More than 30 strokes of the pestle

resulted in lower yield of mitochondria, presumably due to disruption and rupture of the organelle.

9. The supernatant at this step is very turbid, and if the homogenate is too concentrated it will be quite difficult to distinguish the pellet from the supernatant. If this occurs, dilute the homogenate 2–5 fold in MIB and repeat centrifugation.
10. Repeated centrifugations will result in purer mitochondria, but will lower the yield slightly. If high purity is desired do an additional centrifugation.
11. We observed that a “crude” mitochondrial preparation was sufficient for mitochondrial cysteine identification. Further purification only led to a modest improvement in identification, but if non-mitochondrial protein removal is desired proceed with isolation procedure for “pure” mitochondria.
12. A small volume of phenol red can be added to the 52% percoll solution, for easier identification of the 52:26% percoll interface.
13. This step works in both a fixed angle (used in these experiments) as well as swinging bucket rotor.
14. Slide 16-gauge needle down the side of the ultracentrifuge tube, with needle opening facing inward, until it is level with mitochondrial band. Slowly extract band, while moving needle around the edge of the tube. Attempt to remove the band completely, avoiding extracting too much of the percoll solution or any additional visible bands (extracted volume should be less than 0.5 mL).
15. It is essential that the pellets are thoroughly washed with PBS, as Tris will inhibit the CuAAC chemistry from proceeding.
16. When comparing cysteine reactivity from two mitochondrial proteomes it is necessary that the two proteome samples are accurately normalized in terms of total protein concentration.
17. As the mitochondrial proteome is much less complex than the complete mammalian proteome, the standard 2 mg/mL concentration is not necessary and 1 mg/mL is sufficient to get broad coverage of mitochondrial reactive cysteine residues.
18. If cutting membrane in thirds is too cumbersome, multiple SDS-PAGE gels can be run, and each gel used for one western blot. Additional or alternative proteins can be used as readouts of organelle purity at the discretion of the researcher.
19. Intact mitochondria, as opposed to mitochondrial lysates, are labeled with the membrane permeable IA-alkyne in order to preserve sensitive oxidative cysteine modifications that might be lost once the organelle membrane is disrupted. If this is unnecessary, mitochondria can be lysed before IA-alkyne labeling.
20. After the last water wash, completely remove supernatant using a pipette. At this point the beads should have a faint yellow tinge, which indicates enrichment of azo-tagged proteins.

21. After tryptic digestion, the supernatant can be saved as a tryptic peptide fraction and analyzed by LC/LC-MS/MS. This is generally only done when troubleshooting, such as when no IA-labeled peptides are identified by LC/LC-MS/MS upon azobenzene cleavage.
22. Upon addition of sodium hydrosulfite solution, the beads should turn white as an indication of successful azobenzene cleavage.
23. To make a tip, cut ~45 cm of 100 μ m fused-silica capillary tubing, burn off ~ 3 cm of polyimide coating in the center of the capillary tubing, wipe off coating with methanol, and use a laser tip puller to pull two 5 μ m tips. The desalting column is made by attaching a 12-cm length of 250 μ m fused-silica capillary tubing to an inline micro-filter assembly.

5. Results

To demonstrate the utility of the mitochondrial cysteine enrichment platform, “crude” and “pure” intact mitochondria were isolated from HEK293T cells. These were compared to whole cell lysates by western blot analysis of a mitochondrial protein (complex V inhibitor protein), an ER protein (calreticulin), and a cytosolic protein (GAPDH). Increased mitochondrial enrichment was demonstrated by loss of GAPDH and calreticulin signal, for the mitochondrial fractions after each stage of enrichment (Figure 2). Whole cell lysates and “pure” intact mitochondria were IA-labeled, appended to Azo-L tag by CuACC and subject to enrichment and LC/LC-MS/MS analysis in triplicate as described above. The number of mitochondrial reactive cysteine containing peptides and proteins identified from both samples was determined using the mitoMiner resource (<http://mitominer.mrc-mbu.cam.ac.uk>). While only ~ 200 cysteine residues on ~ 100 mitochondrial proteins could be identified in whole cell lysates, those numbers increase to ~ 1,400 cysteine residues on ~ 400 proteins when “pure” intact mitochondria are IA-labeled (Figure 3). This vast improvement in MS identification of mitochondrial reactive cysteine residues demonstrate the utility of our platform for interrogating functional cysteine residues on mitochondrial proteins.

6. Discussion

Global cysteine-profiling has been used to unveil the functional roles of countless cysteine residue on diverse families of proteins, including metal binding sites, sites of oxidative PTMs, active-site nucleophiles, and redox active disulfide bonds. While these functional cysteine residues have been identified on proteins from all subcellular compartments, in general there is poor coverage of proteins from most organelles, including the mitochondria. Here we present a platform to isolate intact mitochondrial for IA-alkyne probe labeling and isoTOP-ABPP analysis. This platform, like standard isoTOP-ABPP performed on whole cell lysates, allows for profiling over a thousand reactive cysteine residues concurrently. As cysteine is susceptible to a number of different oxidative modifications, analysis of protein abundance is not often correlated with activity state. Unlike IA-labeling of whole cell lysates, where reversible oxidative PTMs can easily be lost, by IA-labeling intact mitochondria, the redox state of these cysteine residues is better preserved. This is especially

important as mitochondria are primary sites of ROS production and are subject to fluxes in redox state, that would easily be lost upon lysis. This platform has the potential to inform on the role of cysteine residues in mitochondrial redox signaling under native and (patho)physiological conditions, such as cancer, neurodegeneration, and aging.

Acknowledgments

This work was funded by NIH grant 1R01GM117004 (E.W.).

References

1. Lopez MF, Kristal BS, Chernokalskaya E, Lazarev A, Shestopalov AI, Bogdanova A, Robinson M (2000) High-throughput profiling of the mitochondrial proteome using affinity fractionation and automation. *Electrophoresis* 21:3427–40 [PubMed: 11079563]
2. Bak DW, Weerapana E (2015) Cysteine-mediated redox signalling in the mitochondria. *Mol BioSyst* 11:678–697 [PubMed: 25519845]
3. Wang C, Weerapana E, Blewett MM, Cravatt B (2013) A chemoproteomic platform to quantitatively map targets of lipid-derived electrophiles. *Nat Methods* 11:79–85 [PubMed: 24292485]
4. Pace NJ, Weerapana E (2013) A Competitive Chemical-Proteomic Platform to Identify Zinc-Binding Cysteines. *ACS Chem Biol* 9:258–265 [PubMed: 24111988]
5. Weerapana E, Wang C, Simon GM, Richter F, Khare S, Dillon MB, Bachovchin DA, Mowen K, Baker D, Cravatt BF (2010) Quantitative reactivity profiling predicts functional cysteines in proteomes. *Nature* 468:790–795 [PubMed: 21085121]
6. Zhou Y, Wynia-Smith SL, Couvartier SM, Kalous KS, Marletta MA, Smith BC, Weerapana E (2016) Chemoproteomic strategy to quantitatively monitor transnitrosation uncovers functionally relevant S-nitrosation sites on cathepsin D and HADH2. *Cell Chem Biol* 23:727–37 [PubMed: 27291402]
7. Abo M, Weerapana E (2015) A Caged Electrophilic Probe for Global Analysis of Cysteine Reactivity in Living Cells. *J Am Chem Soc* 137:7087–7090 [PubMed: 26020833]
8. Wabnitz GH, Goursot C, Jahraus B, Kirchgessner H, Hellwig A, Klemke M, Konstandin MH, Samstag Y (2010) Mitochondrial translocation of oxidized cofilin induces caspase-independent necrotic-like programmed cell death of T cells. *Cell Death Dis* 1:e58–69. [PubMed: 21364663]
9. Canet-Avilés RM, Wilson MA, Miller DW, Ahmad R, McLendon C, Bandyopadhyay S, Baptista MJ, Ringe D, Petsko GA, Cookson MR (2004) The Parkinson's disease protein DJ-1 is neuroprotective due to cysteine-sulfinic acid-driven mitochondrial localization. *Proc Natl Acad Sci USA* 101:9103–8 [PubMed: 15181200]
10. Ozawa K, Komatsubara AT, Nishimura Y, Sawada T, Kawafune H, Tsumoto H, Tsuji Y, Zhao J, Kyotani Y, Tanaka T, Takahashi R, Yoshizumi M (2013) S-nitrosylation regulates mitochondrial quality control via activation of parkin. *Sci Rep* 3:2202–2211 [PubMed: 23857542]
11. Yang KS, Kang SW, Woo HA, Hwang SC, Chae HZ, Kim K, Rhee SG (2002) Inactivation of human peroxiredoxin I during catalysis as the result of the oxidation of the catalytic site cysteine to cysteine-sulfinic acid. *J Biol Chem* 277:38029–36
12. McStay GP, Clarke SJ, Halestrap AP (2002) Role of critical thiol groups on the matrix surface of the adenine nucleotide translocase in the mechanism of the mitochondrial permeability transition pore. *Biochem J* 367:541–548 [PubMed: 12149099]
13. Couvartier SM, Zhou Y, Weerapana E (2014) Chemical-proteomic strategies to investigate cysteine posttranslational modifications. *Biochim. Biophys. Acta, Proteins Proteomics* 1844:2315–2330
14. Paulsen CE, Carroll KS (2013) Cysteine-Mediated Redox Signaling: Chemistry, Biology, and Tools for Discovery. *Chem Rev* 113:4633–4679 [PubMed: 23514336]
15. Chouchani ET, Kazak L, Jedrychowski MP, Lu GZ, Erickson BK, Szpyt J, Pierce KA, Laznik-Bogoslavski D, Vetrivelan R, Clish CB, Robinson AJ Gygi SP, Spiegelman BM (2016) Mitochondrial ROS regulate thermogenic energy expenditure and sulfenylation of UCP1. *Nature* 532:112–116 [PubMed: 27027295]

16. Chouchani ET, Methner C, Nadtochiy SM, Logan A, Pell VR, Ding S, James AM, Cochemé HM, Reinhold J, Lilley KS, Partridge L, Fearnley IM, Robinson AJ, Hartley RC, Smith RAJ, Krieg T, Brookes PS, Murphy MP (2013) Cardioprotection by S-nitrosation of a cysteine switch on mitochondrial complex I. *Nat Med* 19:753–759 [PubMed: 23708290]
17. Abo M, Bak DW, Weerapana E (2016) Optimization of Caged Electrophiles for Improved Monitoring of Cysteine Reactivity in Living Cells. *ChemBioChem* 18:81–84 [PubMed: 27813293]
18. Pagliarini DJ, Calvo SE, Chang B, Sheth SA, Vafai SB, Ong SE, Walford GA, Sugiana C, Boneh A, Chen WK, Hill DE, Vidal M, Evans JG, Thorburn DR, Carr SA, Mootha VK (2008) A mitochondrial protein compendium elucidates complex I disease biology. *Cell* 134:112–23. [PubMed: 18614015]
19. Bak DW, Pizzagalli MD, Weerapana E (2017) Identifying Functional Cysteine Residues in the Mitochondria. *ACS Chem Biol* 12:947–957 [PubMed: 28157297]
20. Macpherson LJ, Dubin AE, Evans MJ, Marr F, Schultz PG, Cravatt BF, Patapoutian A (2007) Noxious Compounds Activate TRPA1 Ion Channels through Covalent Modification of Cysteines. *Nature* 445:541–545. [PubMed: 17237762]
21. Qian Y, Martell J, Pace NJ, Ballard TE, Johnson DS, Weerapana E (2013) An Isotopically Tagged Azobenzene-Based Cleavable Linker for Quantitative Proteomics. *Chembiochem* 14:1410–414 [PubMed: 23861326]
22. Frezza C, Cipolat S, Scorrano L (2007) Organelle isolation: functional mitochondria from mouse liver, muscle and cultured fibroblasts. *Nat Protoc* 2:287–295 [PubMed: 17406588]
23. Eng JK, McCormack AL, Yates JR (1994) An approach to correlate tandem mass spectral data of peptides with amino acid sequences in a protein database. *J Am Soc Mass Spectrom* 5:976–89 [PubMed: 24226387]
24. Tabb DL, McDonald WH, Yates JR (2002) DTASelect and Contrast: Tools for Assembling and Comparing Protein Identifications from Shotgun Proteomics. *J Proteome Res* 1:21–26 [PubMed: 12643522]
25. Calvo SE, Clauser KR, Mootha VK (2016) MitoCarta2.0: an updated inventory of mammalian mitochondrial proteins. *Nucleic Acids Res* 44:1251–1257
26. Smith AC, Robinson AJ (2016) MitoMiner v3.1, an update on the mitochondrial proteomics database. *Nucleic Acids Res* 44:D1258–D1261 [PubMed: 26432830]

Figure 1.

The reagents and experimental setup of mitochondrial cysteine enrichment platform. (A) Purification of mitochondria involves the homogenization of cultured cells (Step 1), followed by differential centrifugation (Step 2) and gradient centrifugation (Step 3) to afford “crude” and “pure” mitochondrial fractions, respectively. (B) Intact mitochondria are then IA-alkyne labeled to covalently modify mitochondrial reactive cysteine residues (Step 4). (C) Structure of Azo-L and Azo-H tags. The azo tags contain an azide for click chemistry, an isotopically tagged valine for quantification, an azobenzene cleavable linker, and a biotin for enrichment. (D) After intact mitochondria from two proteomes (A and B) are labeled with IA-alkyne and quickly sonicated, the labeled proteins are then conjugate by CuACC to Azo-L (Proteome A) or Azo-H (Proteome B) tags (Step 5), mixed and enriched on streptavidin beads (Step 6), and subject to on-bead trypsin digestion (Step 7). Labeled peptides are then eluted by sodium hydrosulfite treatment (Step 8) and analyzed by LC/LC-MS/MS using a MudPIT protocol (Step 9), where peptides are identified and quantified (Step 10).

Figure 2.

Western-blot analysis of whole cell, 'crude' and 'pure' mitochondria purity using antibodies against the cytosolic protein, GAPDH, the ER protein, calreticulin, and the mitochondrial protein, complex V inhibitor protein.

Figure 3.

Comparison of the number of identified mitochondrial proteins and peptides from labeling whole cell or "pure" mitochondria with 100 μ M IA-alkyne. Data represent the mean $\pm$ s.d. of three biological replicates.

Table 1.

Solvent systems utilized for MudPIT analysis

Step 1: 0% Buffer C

Time (min)	% Buffer A	% Buffer B	% Buffer C
0.00	100	0	0
5.00	100	0	0
60.00	55	45	0
70.00	0	100	0
100.00	0	100	0

Step 2: 50% Buffer C

Time (min)	% Buffer A	% Buffer B	% Buffer C
0.00	100	0	0
6.00	100	0	0
6.10	45	5	50
8.00	45	5	50
8.10	95	5	0
15.00	85	15	0
35.00	75	25	0
75.00	45	55	0
80.00	45	55	0

Step 3: 50% Buffer C

Time (min)	% Buffer A	% Buffer B	% Buffer C
0.00	100	0	0
5.00	100	0	0
5.10	15	5	80
8.00	15	5	80
8.10	95	5	0
18.00	85	15	0
63.00	75	25	0
115.00	45	55	0
120.00	45	55	0

Step 4: 100% Buffer C

Time (min)	% Buffer A	% Buffer B	% Buffer C
0.00	100	0	0
4.00	100	0	0
4.10	0	0	100
20.00	0	0	100

Step 1: 0% Buffer C

Time (min)	% Buffer A	% Buffer B	% Buffer C
20.10	93	7	0
25.00	85	15	0
100.00	70	30	0
184.00	0	100	0
194.00	0	100	0
195.00	100	0	0
200.00	100	0	0

Step 5: 100% Buffer C

Time (min)	% Buffer A	% Buffer B	% Buffer C
0.00	100	0	0
4.00	100	0	0
4.10	0	0	100
14.00	0	0	100
14.10	93	7	0
30.00	70	30	0
50.00	0	100	0
55.00	0	100	0
56.00	100	0	0
60.00	100	0	0