Published in final edited form as:

Methods Enzymol. 2010 ; 473: . doi:10.1016/S0076-6879(10)73004-4.

Use of Dimedone-Based Chemical Probes for Sulfenic Acid Detection: Methods to Visualize and Identify Labeled Proteins

Kimberly J. Nelson^{*}, Chananat Klomsiri^{*}, Simona G. Codreanu[†], Laura Soito^{*}, Daniel C. Liebler[†], LeAnn C. Rogers^{*}, Larry W. Daniel^{*}, and Leslie B. Poole^{*}

^{*}Department of Biochemistry, Wake Forest University School of Medicine, Winston-Salem, North Carolina, USA

[†]Department of Biochemistry, Vanderbilt University School of Medicine, Nashville, Tennessee, USA

Abstract

Reversible thiol modification is a major component of the modulation of cell-signaling pathways by reactive oxygen species. Hydrogen peroxide, peroxyxynitrite, or lipid hydroperoxides are all able to oxidize cysteines to form cysteine sulfenic acids; this reactive intermediate can be directly reduced to thiol by cellular reductants such as thioredoxin or further participate in disulfide bond formation with glutathione or cysteine residues in the same or another protein. To identify the direct protein targets of cysteine modification and the conditions under which they are oxidized, a series of dimedone-based reagents linked to affinity or fluorescent tags have been developed that specifically alkylate and trap cysteine sulfenic acids. In this chapter, we provide detailed methods using one of our biotin-tagged reagents, DCP-Bio1, to identify and monitor proteins that are oxidized *in vitro* and *in vivo*. Using streptavidin-linked agarose beads, this biotin-linked reagent can be used to affinity capture labeled proteins. Stringent washing of the beads prior to elution minimizes the contamination of the enriched material with unlabeled proteins through coimmunoprecipitation or nonspecific binding. In particular, we suggest including DTT in one of the washes to remove proteins covalently linked to biotinylated proteins through a disulfide bond, except in cases where these linked proteins are of interest. We also provide methods for targeted approaches monitoring cysteine oxidation in individual proteins, global approaches to follow total cysteine oxidation in the cell, and guidelines for proteomic analyses to identify novel proteins with redox sensitive cysteines.

1. Introduction

It is increasingly clear that reversible thiol modification is a major component of the modulation of cell-signaling pathways by reactive oxygen species (ROS). Superoxide anions result from the partial reduction of oxygen by the mitochondrial electron transport chain (Jones, 2006) and are subsequently converted to H₂O₂ either nonenzymatically or by superoxide dismutase. ROS are also generated by activated forms of the enzymes NADPH oxidase, xanthine oxidase, cyclooxygenase, and lipoxygenase (Schneider *et al.*, 2007; Thomas *et al.*, 2008). In general, the initial product of cysteine oxidation by H₂O₂, peroxyxynitrite, or lipid hydroperoxides is cysteine sulfenic acid, suggesting a pivotal importance for this modification in the response of proteins to ROS. Sulfenic acid-modified proteins can be directly reduced to thiol by cellular reductants or further participate in disulfide bond formation with glutathione or cysteine residues in the same or another protein.

Most evidence of catalytic or regulatory sulfenic acid formation has come from individual studies of purified proteins including peroxiredoxins, organic hydroperoxide resistance

protein (Ohr), NADH peroxidase, and methionine sulfoxide reductase (Claiborne *et al.*, 1999; Poole *et al.*, 2004). The redox status of cysteine has been shown to regulate cellular metabolism through oxidation of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Cotgreave *et al.*, 2002; Schmalhausen *et al.*, 1999) and MetE (Hondorp and Matthews, 2004), and to regulate the molecular chaperone activity of HSP33 (Jakob *et al.*, 1999) and the activity of protein tyrosine phosphatases including PTP1B and PTEN (Tonks, 2005). Oxidative cysteine modifications have also been shown to control transcriptional regulation by OxyR (Zheng *et al.*, 1998), OhrR (Fuangthong and Helmann, 2002; Hong *et al.*, 2005; Panmanee *et al.*, 2006), RsrA (Kang *et al.*, 1999), Yap1p (Kuge *et al.*, 2001), and p53 (Rainwater *et al.*, 1995).

Because cysteine sulfenic acid is unstable in many proteins, studies have been limited by the ability to monitor and identify proteins and sites that have redox-regulated cysteines. To fill this gap, our labs and other groups have developed a series of reagents, based on dimedone, that specifically alkylate and, therefore, trap cysteine sulfenic acids; these chemical probes are or can be linked to affinity or fluorescent tags (Fig. 4.1) (Leonard *et al.*, 2009; Poole and Nelson, 2008; Poole *et al.*, 2007; Reddie *et al.*, 2008). These reagents are designed to enable enrichment and sensitive detection of proteins or peptides bearing sulfenic acid modifications. Though sulfenic acids likely represent a dynamic and transient oxidation product, their unique chemistry allows them to be captured by dimedone-based labeling reagents before progression to a potentially more complex array of disulfide-bonded or oxidized products. Even rapid “trapping” of sulfenic acids in proteins is still likely to yield substoichiometric amounts of label incorporated into given proteins due to the generally transient nature of the modification. Reliable quantification based on extent of probe incorporation is likely to be difficult to achieve, though large time-dependent responses in oxidation for individual cellular proteins may be observed across samples within the same experimental set. Despite these challenges, these probes and techniques represent an important step forward in identifying cysteine residues that are oxidized in cells in response to a particular stimulant. As with phosphorylation sites, all oxidation sites are not expected to have the same role (or for that matter any role) in modulating protein function. Not only may cysteine oxidation be stimulatory, inhibitory, or inconsequential to the activity, stability, or localization of a given protein, but sulfenic acids also have the capacity to go on to form multiple products depending on cellular context that may themselves have different functional effects (Poole and Nelson, 2008). The true biochemical evaluation of these sites must ultimately involve both mapping the specific residue being oxidized and evaluating the effect of these oxidations on protein function.

We report here some of the potential applications of these sulfenic acid-specific reagents and provide protocols for visualizing and identifying labeled proteins. The previous chapter in this volume provides detailed protocols and controls for labeling both pure and cellular proteins with these probes (Klomsiri *et al.*, 2010). Although we have developed and used a variety of probes containing fluorescent groups such as rhodamine and fluorescein (Klomsiri *et al.*, 2010; Poole *et al.*, 2007), we have found that biotin-linked reagents are particularly powerful tools because they allow for affinity capture of the labeled proteins and/or peptides. In this chapter, we provide detailed methods using one of our biotin-tagged reagents, DCP-Bio1 (Fig. 4.1) to identify and monitor proteins that are oxidized *in vitro* and *in vivo*. We also provide methods to monitor global changes in cysteine sulfenic acid formation.

2. Biotin-Based Affinity Capture to Identify Proteins Containing Cysteine Sulfenic Acids

The greatest advantage of the biotin-conjugated, sulfenic acid-directed reagents is the ability to use affinity methods to capture the labeled proteins. Because the captured material will be used in many cases to identify labeled proteins and determine the extent of protein labeling, it is very important to ensure that essentially all proteins in the affinity-enriched material contain the biotin modification and are not simply coprecipitating with the labeled proteins. Thus, we have developed a protocol to stringently wash the streptavidin beads prior to elution. In particular, including DTT in one of the washes ensures that unlabeled proteins covalently linked to biotinylated proteins through a disulfide bond will not be included in the eluted material.

2.1. Materials

2.1.1. Solutions

1. 500 mM biotin maleimide in dimethyl sulfoxide (DMSO)
2. 100 mM 1,4-dithio-DL-threitol (DTT) in dH₂O
3. 100 mM diethylene triamine pentaacetic acid (DTPA) in 1 M sodium hydroxide
4. 25 mM potassium phosphate, pH 7.0, 100 μ M DTPA
5. Empty spin columns with ~1.2-ml bed volume (e.g., Bio-Spin Chromatography columns from Bio-Rad)
6. Bio-Gel P6 desalting resin (Bio-Rad); 1 ml resin is loaded into empty spin column and equilibrated with 3 column volumes (CV) of buffer
7. Spin column with screw cap and column plug – 500- μ l bed volume (e.g., Pierce Spin Columns – screw cap with Luer-Lok adaptors)
8. Streptavidin beads (e.g., Pierce High Capacity Streptavidin–Agarose Resin)
9. Sepharose CL-4B (or other resin similar to the streptavidin support material)
10. Phosphate-buffered saline (PBS): 100 mM sodium phosphate, 150 mM sodium chloride, pH 7.2
11. 8 M urea in PBS
12. Wash solution 1: 1% SDS in dH₂O
13. Wash solution 2: 4 M urea in PBS
14. Wash solution 3: 1 M NaCl in dH₂O
15. Wash solution 4: 100 μ M ammonium bicarbonate with 10 mM DTT
16. Wash solution 5: 100 μ M ammonium bicarbonate
17. Deionized H₂O (dH₂O)
18. Elution buffer for 1D gel electrophoresis: 2% SDS, 50 mM Tris–HCl, 1 mM EDTA, pH 8.0
19. Elution buffer for 2D gel electrophoresis: 8 M urea, 2% CHAPS

2.1.2. Proteins

1. Protein samples labeled with biotin-linked reagent specific for sulfenic acid (e.g., DCP-Bio1) (Poole *et al.*, 2007); see accompanying chapter for suggested labeling protocols (Klomsiri *et al.*, 2010).
2. *Salmonella typhimurium* AhpC C165S mutant, purified as described previously (Nelson *et al.*, 2008; Poole and Ellis, 1996) and stored at -20°C in 5 mM DTT. Prior to conducting experiments, DTT is removed using a Bio-Gel P6 spin column equilibrated in 25 mM potassium phosphate, pH 7.0, 100 μM DTPA. Alternatively, any other pure protein containing at least one cysteine residue can be used.

2.2. Methods

2.2.1. Use of biotinylated AhpC as a procedural control for affinity capture of biotinylated samples

—To ensure that changes to biotinylation levels between a set of samples is due to physiological changes and not variation in the efficiency of the affinity capture and elution procedure, we add a biotinylated control protein to each sample before affinity capture. We typically use a biotinylated version of the C165S mutant of *S. typhimurium* AhpC because large amounts of this recombinant protein can be purified in *Escherichia coli* and because the presence of only one cysteine allows for stoichiometric labeling. This protein is stored in 5 mM DTT, which is removed using a desalting column (i.e., PD10 or Bio-Gel P6). To do this, spin columns (1.2-ml bed volume) containing ~ 1 ml Bio-Gel P6 Resin are equilibrated with 25 mM potassium phosphate, 100 μM DTPA, pH 7.0, centrifuged for 25 min at $1000\times g$, and moved to clean, labeled microfuge tubes. Samples are applied to the top, the column is centrifuged for 2 min at $1000\times g$, then the material from the flow-through is supplemented with biotin maleimide from a 200 mM stock to a final concentration of 10 mM (DMSO concentration in final solution should be no more than 2%) and incubated at room temperature overnight. Excess biotin maleimide is removed using a second desalting column equilibrated in 25 mM potassium phosphate with 100 μM DTPA, pH 7.0, and the concentration of C165S AhpC is determined based upon absorbance at 280 nm ($\epsilon = 24,300 \text{ M}^{-1} \text{ cm}^{-1}$). Using this procedure, C165S AhpC was fully labeled with biotin maleimide based upon matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometry (MS) analysis.

Any pure protein can be used for this purpose as long as it contains at least one Cys residue and is not present in the sample to be analyzed. For proteins not stored in DTT, 10 mM DTT should first be added and incubated for 30 min at room temperature prior to the first desalting column and alkylation with biotin maleimide.

2.2.2. Sample preparation to remove unreacted DCP-Bio1 and to add control protein

—Cell lysates are labeled with DCP-Bio1 (Poole *et al.*, 2007) according to the protocol described in the preceding chapter (Klomsiri *et al.*, 2010). In order to prevent further biotinylation of proteins due to nonphysiological oxidation and to prevent free DCP-Bio1 from competing with biotinylated protein for binding to the streptavidin binding sites on the resin, proteins in the cell lysate are separated from small molecules immediately upon thawing. Columns containing ~ 1 ml Bio-Gel P6 resin are equilibrated with 2 CV PBS and 2 CV 2 M urea in PBS, pH 7.2. Columns are centrifuged for 2 min at $1000\times g$ and moved to clean, labeled microfuge tubes. Cell lysates are applied to the top of each 1-ml column (if samples are more than 0.2 ml, multiple columns are used). Samples are centrifuged for 2 min at $1000\times g$ and the flow-through material contains the small molecule-free protein. Protein concentrations of each sample are measured using an appropriate assay, such as the BCA Protein Assay (Pierce) or the DC Protein Assay (Bio-Rad). Samples are diluted to 1

mg/ml with urea to a final concentration of 2 *M*, and biotinylated C165S AhpC (1 μ g/500 μ g of cell lysate) is added as a procedural control (prepared as described above).

2.2.3. Capture of biotinylated proteins with stringent washing to prevent coprecipitation of unlabeled proteins—For each sample, one spin column (0.5-ml bed volume with screw cap lid) is prepared with nonliganded support beads (i.e., cross-linked agarose, e.g., Sepharose CL-4B) to remove proteins with a tendency to bind nonspecifically to such beads; a second column with streptavidin–agarose beads is also prepared. The binding capacity of the streptavidin beads is used to determine the resin volume needed for the amount of protein being enriched. For Sepharose CL-4B (Sigma) and High Capacity Streptavidin–Agarose Resin (Pierce), 80 μ l of resin is used for a typical sample containing 400 μ g cell lysate. For HEK293 cells, 400 μ g cellular protein can typically be obtained from two 75% confluent, 100-mm dishes harvested with 150 μ l lysis buffer per plate. Both columns are equilibrated with 20 CV 2 *M* urea in PBS, pH 7.2. Immediately prior to adding sample, columns are placed in 1.5-ml microfuge tubes, then centrifuged for 2 min at 1000 $\times$ *g*.

To remove proteins that might bind nonspecifically to the beads, prepared cell lysates are added to the plugged columns containing Sepharose CL-4B beads, the tops are closed with a screw cap lid, columns are sealed into a clean microfuge tube with Parafilm to prevent leaks, and the samples are incubated for 2 h at 4 °C with constant rotation. Parafilm and plugs are removed and columns are returned to the tubes, then centrifuged for 1000 $\times$ *g* for 2 min. The flow-through from these columns is transferred to plugged columns containing streptavidin–agarose beads, capped, and sealed into microfuge tubes, and rotated for ~16 h at 4 °C. Column assemblies are centrifuged at 1000 $\times$ *g* for 2 min to remove unbound proteins.

Beads bound to the biotinylated proteins are washed twice with 4 CV 1% SDS, twice with 4 CV 4 *M* urea, and twice with 4 CV 1 *M* NaCl. For each of these solutions, the first wash is incubated with constant rotation for 30 min at 4 °C and the second wash is applied for 1 min at room temperature prior to centrifugation. These are followed by additional successive washes at room temperature, once with 4 CV 10 mM DTT in 100 μ M ammonium bicarbonate for 5 min, once with 100 μ M ammonium bicarbonate without DTT, and twice with 4 CV dH₂O. For each wash, the beads are resuspended in the wash solution, rotated for the indicated amount of time, centrifuged at 1000 $\times$ *g* for 2 min, and the supernatant is discarded. The last dH₂O wash is used to remove any resin from the lid of the column.

2.2.4. Elution conditions depend on the downstream application—Although streptavidin has a high affinity for biotin, various elution conditions can be used (Fig. 4.2) and should be selected based upon the intended downstream application. We recommend use of an SDS-containing sample buffer for 1D gel electrophoresis. First, the bottom of the column is stoppered, 2.5 CV of 2% SDS in 50 mM Tris–HCl, 1 mM EDTA pH 8.0 is added, the cap is replaced loosely to prevent leakage due to pressure build up, the column is placed inside a clean microfuge tube to collect any sample that might leak, and the whole assembly is heated to 90 °C for 10 min (Fig. 4.2 A). For 2D gel electrophoresis, we recommend adding 2.5 CV of 8 *M* urea, 2% CHAPS, and 50 mM DTT to the plugged and loosely capped column and heating for 20 min at 37 °C, shaking periodically. If the presence of SDS or urea is problematic for downstream applications (e.g., for MS experiments), alternate elution conditions include incubation with 100 mM glycine, pH 2.8 for 10 min at room temperature (Fig. 4.2 A) or proteolytic digestion of the sample while still bound to the streptavidin beads. Because DCP-Bio1 contains an ester linkage, labeled proteins containing the DCP functional group can be cleaved from the biotin moiety by incubation with 2.25 *M* ammonium hydroxide for ~16 h at 24 °C (Fig. 4.2 B). For all elution conditions, the spin column is centrifuged at 1000 $\times$ *g* for 3 min and the flow-through is retained.

3. Detection Methods to Identify Oxidized Proteins and Cysteines

Potential uses for sulfenic acid-specific trapping agents include monitoring of cysteine oxidation of a particular protein under various conditions (targeted approaches), following cysteine oxidation of total cellular proteins in response to various stimuli (global approaches), and identifying novel proteins with redox sensitive cysteine residues (proteomic approaches). In this section, we provide protocols suitable for all of these approaches. Although we briefly touch on techniques including Western blots, immunoprecipitation (IP), and MS analysis, detailed protocols for these are only given for the proteins studied herein, and we focus on how these techniques can be adapted to identify and monitor proteins labeled with DCP-Bio1 and related compounds.

3.1. Targeted approaches: Western blot of affinity-enriched proteins to analyze proteins of interest

3.1.1. Materials

1. DCP-Bio1-labeled samples after affinity capture of biotinylated proteins
2. Materials for 1D gel electrophoresis and transfer to nitrocellulose (or PVDF) membrane
3. TBST buffer: 50 mM Tris-HCl, 150 mM NaCl, 0.1% (v/v) Tween-20, pH 7.5
4. 5% (w/v) dried milk in TBST (alternate blocking and binding buffer may be preferred for individual antibodies; commonly used buffers include 1–5% bovine serum albumin (BSA) or 1–10% fetal bovine serum (FBS))
5. Primary antibody to protein of interest
6. Secondary antibody conjugated to horseradish peroxidase (HRP) (other conjugates such as alkaline phosphatase or fluorescein may also be used)
7. Chemiluminescence detection kit (such as Western Lightning® Plus-ECL, Enhanced Chemiluminescence Substrate from Perkin Elmer or Pico, Dura, or Femto Chemiluminescence kits from Pierce)

3.1.2. Methods—Western blots are performed on equal volumes of biotinylated, affinity-enriched samples according to the established protocol for each antibody and target protein. Although different proteins and samples will require optimization of conditions, we have found that the amount of affinity-captured material from 40 μ g starting lysate is typically sufficient for visualization using the Dura chemiluminescence kit from Pierce.

Time-dependent changes in protein sulfenic acid levels in HEK293 cells, analyzed by Western blot analyses of the affinity-captured material, show that phosphatases (PTEN and SHP-2), kinases (protein kinase C, PKC-1), chaperones (heat shock protein, HSP70), and glycolytic enzymes (GAPDH) are all transiently oxidized and labeled by DCP-Bio1 in tumor necrosis factor- α (TNF α) treated cells (Fig. 4.3). Some of the proteins we observe, such as GAPDH, are known to form a stabilized sulfenic acid, and others (PTEN, SHP-2, and HSP70) are known to be redox regulated, though not specifically through sulfenic acid formation. The signaling phosphatases PTEN (Kwon *et al.*, 2004; Lee *et al.*, 2002) and SHP-2 (Chen *et al.*, 2006; Kwon *et al.*, 2005; Meng *et al.*, 2002) are known to be oxidation sensitive, with the former yielding an intrasubunit disulfide bond between the active site Cys (Cys124) and Cys71; sulfenic acid formation has been postulated based on other protein tyrosine phosphatases (Tonks, 2005), but not fully demonstrated in each case. In the case of HSP70, protein S-glutathionylation in retinal pigment epithelium has been shown to convert this protein to an active chaperone (Hoppe *et al.*, 2004).

We note from our blots (Fig. 4.3) that patterns of oxidation differ between proteins in the same experimental samples, suggesting distinct influences on oxidation status of various proteins after cell stimulation. Because each protein exhibits a different profile (Fig. 4.3), we typically select a range of times between 1 min and 1 h after TNF stimulation to maximize the chance of observing the transient intermediate on a particular protein.

3.2. Targeted approaches: Immunoprecipitation of protein of interest followed by Western blot to detect biotin

3.2.1. Materials

1. DCP-Bio1-labeled samples prior to affinity enrichment
2. Primary antibody to protein of interest (suitable for IP)
3. Secondary antibody complexed to agarose or magnetic beads
4. 2 M urea in PBS
5. Elution buffer for 1D gel electrophoresis: 2% SDS, 50 mM Tris-HCl, 1 mM EDTA, pH 8.0
6. Materials for 1D gel electrophoresis and transfer to nitrocellulose (or PVDF) membrane
7. Antibiotin antibody and HRP-conjugated secondary antibody (or streptavidin-HRP conjugate)
8. 5% (w/v) dried milk in TBST
9. 1% BSA in TBST

3.2.2. Methods—The previous section described performing a Western blot on the affinity-captured biotinylated samples to look for the target protein; an alternate and complementary approach is to immunoprecipitate the target protein, then probe the samples for biotin. Use of both techniques gives the strongest evidence that a given protein is indeed labeled by the biotinylated reagent as long as proper controls in the absence of reagent are included.

IP is performed according to established protocols specific to the protein of interest. After beads have been incubated with the protein samples, they are washed with PBS or more stringent conditions (if possible), then eluted with SDS sample buffer. Biotin blots are then performed on the eluted, electrophoresed samples using either HRP-conjugated streptavidin or antibiotin antibodies. In our hands, antibiotin antibodies perform somewhat better. In order to avoid coelution of the antibody proteins, antibodies may be cross-linked to the beads prior to IP. Otherwise, a control for the antibody without lysate should be included as we have observed biotinylation of the antibody subunits and these can mask detection of the target protein if of similar size.

Using this approach, we were able to clearly demonstrate the presence of biotinylated proteins at the correct molecular weight in the IPs of PTEN and SHP-2 proteins (Fig. 4.4), confirming the data using the affinity-captured material that identified these two proteins as sulfenic acid-containing proteins. For IP experiments shown in Fig. 4.4, TNF -treated HEK293 lysates were preincubated with 20 μ l Dynabeads-Protein A magnetic beads preequilibrated in PBS and then incubated overnight at 4 °C with 0.5 μ l of either anti-PTEN or anti-SHP-2 antibody (Cell Signaling) and 20 μ l Dynabeads-Protein A magnetic beads. The supernatant was then removed and the beads were washed three times with PBS buffer.

Immunoprecipitated proteins were eluted with 35 μ l SDS–PAGE-loading buffer and biotinylated proteins were visualized using Streptavidin–HRP.

3.3. Controls for endogenous biotinylation

The biotin affinity capture and detection procedures described herein (with the exception of MS analysis) cannot distinguish between proteins labeled with a biotinylated chemical reagent and those that are endogenously biotinylated on lysine. To test for endogenous biotinylation, it is important to include a sample that has not been labeled with the biotin compound as a control in all experiments (Fig. 4.4).

3.4. Global approaches: Identification of overall sulfenic acid levels for cellular proteins in response to stimuli

In order to estimate the relative abundance of proteins containing sulfenic acid in the cellular proteome under various conditions, we have used both biotinylated and fluorescent reagents. In addition to the affinity-based methods employed above, global biotin incorporation can be visualized using gel-based techniques such as total protein staining of gels following affinity capture of biotinylated proteins (Fig. 4.5 A) and anti-biotin Western blots using either a streptavidin–HRP conjugate or an anti-biotin antibody (Fig. 4.5 B). We have also labeled lysates with DCP-FL1, a dimedone analogue containing a fluorescein moiety (as well as other fluorescent compounds including DCP-FL2, DCP-Rho1, and DCP-Rho2) and have visualized sulfenic acid-modified proteins both in 1D (not shown) and 2D gels using a fluorescence imager (Fig. 4.5 C). Finally, we have used a FluoReporter biotin incorporation assay to evaluate total biotin incorporation into cellular proteins (Fig. 4.5 D).

For gel-based analyses of proteins labeled with fluorescent analogues, it is often preferable to first remove excess reagent either by passing lysates through a Bio-Gel P6 spin column, or by precipitating proteins and washing the pellets before resuspension into sample buffer. After electrophoresis, gels can be fixed with 10% acetic acid, 40% methanol and washed at least three times with water prior to visualization. For fluorophores such as fluorescein which are less fluorescent at low pH, fixed gels are washed two times for 15 min each with 40 mM ammonium bicarbonate, pH 8.0.

For separation of either biotinylated or fluorescently labeled proteins by 2D electrophoresis, it is important to ensure that samples not contain any excess reagents or salts as either of these may interfere with isoelectric focusing. Therefore, samples (up to 200 μ l) are precipitated with 1 ml cold acetone, incubated on ice for 10 min and centrifuged at 14,000 $\times$ g for 10 min at 4 °C. Supernatants are removed, the pellets are washed with 0.5 ml 10% trichloroacetic acid, then samples are centrifuged again and washed one more time with 200 μ l of 1:1 ether:ethanol. Liquid is removed and pellets are allowed to air dry prior to the addition of an appropriate buffer for isoelectric focusing (e.g., 8 M urea 2% CHAPS). Sample resuspension is allowed to proceed at 37 °C for 20–60 min with occasional tapping. Protein content is measured, then ampholytes and DTT (10 μ l of a freshly prepared 1 M stock per 200 μ l final volume) are added to the samples just prior to their application onto isoelectric focusing strips. For 2D gels of affinity-captured, biotinylated samples, we typically use samples derived from 0.4 to 1 mg of starting lysate.

We have used the FluoReporter® biotin assay kit from Invitrogen to evaluate biotin incorporation into total protein, an assay which measures the increase in fluorescence as biotin displaces quencher dye from the biotin binding sites on their Biotective™ Green reagent. For this and all assays designed to measure biotin, unreacted DCP-Bio1 and other small molecules must first be removed using a Bio-Gel P6 spin column and the samples exchanged into PBS or another buffer compatible with the assay of interest. Following the

directions in the kit, cell lysates (~10–25 μg total protein in 50- μl volume) are digested with a protease to disrupt protein structure and allow protein-bound biotin to access the binding sites on the dye (Fig. 4.5 D). The FluoReporter assay is able to detect between 4 and 80 pmol of biotin per 50- μl sample, and the digestion mixture will most likely need to be diluted for samples to be in the correct range; for our experiments, 1–2 μg total protein per sample was a good starting amount. A standard curve is generated using a small molecule biotin provided in the kit; the standard curve should include 2–3 replicates of a zero and a minimum of four other biotin concentrations. Analysis of a biotinylated protein used as a positive control is also recommended in order to confirm that proteolysis is sufficient to yield assay-accessible biotin. Each sample is incubated with 50 μl of Biotective Green reagent in a 96-well plate for 5 min at room temperature in the dark. Sample fluorescence is measured on a fluorescence plate reader using $\lambda_{\text{ex}} = 485 \text{ nm}$ and $\lambda_{\text{em}} = 530 \text{ nm}$.

For all of these assays, it is important to gauge the endogenous levels of biotinylation on lysine. Studies in our laboratory to date indicate that these levels are very low when compared with the amount of DCP-Bio1 incorporation, but some prominent bands are present even when no biotin is added (see Fig. 3.5B in the preceding chapter, Klomsiri *et al.*, 2010).

3.5. Global approaches: Identification of oxidized proteins by mass spectrometry after biotin affinity capture

Biotinylated samples can be used for proteomic level analyses in order to identify unknown proteins with peroxide-sensitive cysteines. In order to minimize false positives and to simplify analysis, we suggest performing affinity capture along with stringent washing procedures on such biotinylated samples. For these types of studies, the affinity-captured material is generally separated by 1D or 2D electrophoresis followed by in-gel proteolysis and standard MS analyses to identify the proteins (Shevchenko *et al.*, 2006) Table 4.1 lists a selection of proteins that we have identified from lysates, derived from TNF α -treated HEK-293 cells, using biotin-based affinity capture, separation by either 1D or 2D gel electrophoresis, in-gel proteolytic digestion, and MS analysis.

3.6. Identification of oxidized cysteine by MS–MS analysis

Although stringent washing of the streptavidin beads may help minimize the identification of false positives, the ultimate proof that a protein truly contains an oxidized cysteine comes from the direct identification of the oxidized cysteine. For this purpose, affinity capture of biotinylated peptides can be performed after proteolytic digestion of the cellular proteins (Dennehy *et al.*, 2006; Shin *et al.*, 2007). Alternatively, biotinylated proteins can first be enriched by affinity purification, then digested and further enriched for biotinylated peptides using a second affinity capture step. We have used the first type of experiment to identify the labeled cysteine residue in peroxiredoxin VI using a DCP-Bio1-treated lysate of HEK-293 cells stimulated with 12-*O*-tetradecanoylphorbol-13-acetate (TPA). This peptide was identified by performing a tryptic digest, biotin-based affinity capture of the labeled peptides, and LC–MS/MS analysis (Fig. 4.6). Because this method is only expected to capture 1 or a few peptides per protein, the results will be heavily influenced by the choice of protease. For this reason, it may be desirable to use more than one protease for each sample in order to increase the number of peptides identified.

4. Summary

Reagents developed recently by us and others have provided a new set of tools to specifically trap the largely unstable protein sulfenic acid intermediate in cellular proteins. Using protocols optimized to minimize the presence of unlabeled proteins in our affinity-

captured material, it is now possible to identify proteins and specific cysteine residues that are oxidized in response to cellular stimulation and to monitor the conditions under which these oxidations occur. The protocols presented here are suitable for the large-scale identification of these modified proteins by high-throughput proteomics methods, yet can also be employed to investigate the oxidation of a particular protein or system by a lab with limited access to MS. Although the presence and location of an oxidized cysteine often does not directly indicate whether it modulates protein function, it is an important first step. Once the oxidized cysteine has been identified, future experiments can be designed that explore the role of these oxidations on protein function and cellular processes.

Acknowledgments

This work was supported by NIH grants R21 CA112145 and R33 CA126659 to LBP and by an Established Investigator Grant to LBP from the American Heart Association. Support from a Ruth L. Kirschstein Individual Fellowship from the National Institutes of Health to KJN (F32 GM074537) and a grant from the NSF-NIGMS Program in Mathematical Biology to Jacquelyn S. Fetrow (RO1 GM075304) with LBP and LWD as coinvestigators is also acknowledged.

References

- Chen CH, Cheng TH, Lin H, Shih NL, Chen YL, Chen YS, Cheng CF, Lian WS, Meng TC, Chiu WT, Chen JJ. Reactive oxygen species generation is involved in epidermal growth factor receptor transactivation through the transient oxidization of Src homology 2-containing tyrosine phosphatase in endothelin-1 signaling pathway in rat cardiac fibroblasts. *Mol Pharmacol*. 2006; 69:1347–1355. [PubMed: 16391241]
- Claiborne A, Yeh JI, Mallett TC, Luba J, Crane EJ 3rd, Charrier V, Parsonage D. Protein-sulfenic acids: Diverse roles for an unlikely player in enzyme catalysis and redox regulation. *Biochemistry*. 1999; 38:15407–15416. [PubMed: 10569923]
- Cotgreave IA, Gerdes R, Schuppe-Koistinen I, Lind C. S-glutathionylation of glyceraldehyde-3-phosphate dehydrogenase: Role of thiol oxidation and catalysis by glutaredoxin. *Methods Enzymol*. 2002; 348:175–182. [PubMed: 11885270]
- Dennehy MK, Richards KA, Wernke GR, Shyr Y, Liebler DC. Cytosolic and nuclear protein targets of thiol-reactive electrophiles. *Chem Res Toxicol*. 2006; 19:20–29. [PubMed: 16411652]
- Fuangthong M, Helmann JD. The OhrR repressor senses organic hydro-peroxides by reversible formation of a cysteine-sulfenic acid derivative. *Proc Natl Acad Sci USA*. 2002; 99:6690–6695. [PubMed: 11983871]
- Hondorp ER, Matthews RG. Oxidative stress inactivates cobalamin-independent methionine synthase (MetE) in *Escherichia coli*. *PLoS Biol*. 2004; 2:e336. [PubMed: 15502870]
- Hong M, Fuangthong M, Helmann JD, Brennan RG. Structure of an OhrR-ohrA operator complex reveals the DNA binding mechanism of the MarR family. *Mol Cell*. 2005; 20:131–141. [PubMed: 16209951]
- Hoppe G, Chai YC, Crabb JW, Sears J. Protein s-glutathionylation in retinal pigment epithelium converts heat shock protein 70 to an active chaperone. *Exp Eye Res*. 2004; 78:1085–1092. [PubMed: 15109915]
- Jakob U, Muse W, Eser M, Bardwell JC. Chaperone activity with a redox switch. *Cell*. 1999; 96:341–352. [PubMed: 10025400]
- Jones DP. Disruption of mitochondrial redox circuitry in oxidative stress. *Chem Biol Interact*. 2006; 163:38–53. [PubMed: 16970935]
- Kang JG, Paget MS, Seok YJ, Hahn MY, Bae JB, Hahn JS, Kleanthous C, Buttner MJ, Roe JH. RsrA, an anti-sigma factor regulated by redox change. *EMBO J*. 1999; 18:4292–4298. [PubMed: 10428967]
- Klomsiri C, Nelson KJ, Bechtold E, Soito L, Johnson LC, Todd Lowther W, Seong-Eon Ryu S, King B, Furdui CM, Poole LB. Use of dimedone-based chemical probes for sulfenic acid detection. Evaluation of conditions affecting probe incorporation into redox-sensitive proteins. *Methods Enzymol*. 2010; 473:77–93. [PubMed: 20513472]

- Kuge S, Arita M, Murayama A, Maeta K, Izawa S, Inoue Y, Nomoto A. Regulation of the yeast Yap1p nuclear export signal is mediated by redox signal-induced reversible disulfide bond formation. *Mol Cell Biol.* 2001; 21:6139–6150. [PubMed: 11509657]
- Kwon J, Lee SR, Yang KS, Ahn Y, Kim YJ, Stadtman ER, Rhee SG. Reversible oxidation and inactivation of the tumor suppressor PTEN in cells stimulated with peptide growth factors. *Proc Natl Acad Sci USA.* 2004; 101:16419–16424. [PubMed: 15534200]
- Kwon J, Qu CK, Maeng JS, Falahati R, Lee C, Williams MS. Receptor-stimulated oxidation of SHP-2 promotes T-cell adhesion through SLP-76-ADAP. *EMBO J.* 2005; 24:2331–2341. [PubMed: 15933714]
- Lee SR, Yang KS, Kwon J, Lee C, Jeong W, Rhee SG. Reversible inactivation of the tumor suppressor PTEN by H₂O₂. *J Biol Chem.* 2002; 277:20336–20342. [PubMed: 11916965]
- Leonard SE, Reddie KG, Carroll KS. Mining the thiol proteome for sulfenic acid modifications reveals new targets for oxidation in cells. *ACS Chem Biol.* 2009; 4:783–799. [PubMed: 19645509]
- Meng TC, Fukada T, Tonks NK. Reversible oxidation and inactivation of protein tyrosine phosphatases in vivo. *Mol Cell.* 2002; 9:387–399. [PubMed: 11864611]
- Nelson KJ, Parsonage D, Hall A, Karplus PA, Poole LB. Cysteine pK_a values for the bacterial peroxiredoxin AhpC. *Biochemistry.* 2008; 47:12860–12868. [PubMed: 18986167]
- Panmanee W, Vattanaviboon P, Poole LB, Mongkolsuk S. Novel organic hydroperoxide-sensing and responding mechanisms for OhrR, a major bacterial sensor and regulator of organic hydroperoxide stress. *J Bacteriol.* 2006; 188:1389–1395. [PubMed: 16452421]
- Poole LB, Ellis HR. Flavin-dependent alkyl hydroperoxide reductase from *Salmonella typhimurium* 1 Purification and enzymatic activities of overexpressed AhpF and AhpC proteins. *Biochemistry.* 1996; 35:56–64. [PubMed: 8555198]
- Poole LB, Nelson KJ. Discovering mechanisms of signaling-mediated cysteine oxidation. *Curr Opin Chem Biol.* 2008; 12:18–24. [PubMed: 18282483]
- Poole LB, Karplus PA, Claiborne A. Protein sulfenic acids in redox signaling. *Annu Rev Pharmacol Toxicol.* 2004; 44:325–347. [PubMed: 14744249]
- Poole LB, Klomsiri C, Knaggs SA, Furdai CM, Nelson KJ, Thomas MJ, Fetrow JS, Daniel LW, King SB. Fluorescent and affinity-based tools to detect cysteine sulfenic acid formation in proteins. *Bioconjug Chem.* 2007; 18:2004–2017. [PubMed: 18030992]
- Rainwater R, Parks D, Anderson ME, Tegtmeyer P, Mann K. Role of cysteine residues in regulation of p53 function. *Mol Cell Biol.* 1995; 15:3892–3903. [PubMed: 7791795]
- Reddie KG, Seo YH, Muse WB III, Leonard SE, Carroll KS. A chemical approach for detecting sulfenic acid-modified proteins in living cells. *Mol Biosyst.* 2008; 4:521–531. [PubMed: 18493649]
- Schmalhausen EV, Nagradova NK, Boschi-Muller S, Branlant G, Mironetz VI. Mildly oxidized GAPDH: the coupling of the dehydrogenase and acyl phosphatase activities. *FEBS Lett.* 1999; 452:219–222. [PubMed: 10386594]
- Schneider C, Pratt DA, Porter NA, Brash AR. Control of oxygenation in lipoxygenase and cyclooxygenase catalysis. *Chem Biol.* 2007; 14:473–488. [PubMed: 17524979]
- Shevchenko A, Tomas H, Havlis J, Olsen JV, Mann M. In-gel digestion for mass spectrometric characterization of proteins and proteomes. *Nat Protoc.* 2006; 1:2856–2860. [PubMed: 17406544]
- Shin NY, Liu Q, Stamer SL, Liebler DC. Protein targets of reactive electrophiles in human liver microsomes. *Chem Res Toxicol.* 2007; 20:859–867. [PubMed: 17480101]
- Thomas SR, Witting PK, Drummond GR. Redox control of endothelial function and dysfunction: molecular mechanisms and therapeutic opportunities. *Anti-oxid Redox Signal.* 2008; 10:1713–1765.
- Tonks NK. Redox redux: Revisiting PTPs and the control of cell signaling. *Cell.* 2005; 121:667–670. [PubMed: 15935753]
- Zheng M, Aslund F, Storz G. Activation of the OxyR transcription factor by reversible disulfide bond formation. *Science.* 1998; 279:1718–1721. [PubMed: 9497290]

Figure 4.1.

Reaction of DCP-Bio1 with cysteine sulfenic acid. The sulfenic acid-reactive reagent designated DCP-Bio1 [3-(2,4-dioxocyclohexyl)propyl 5-((3a*R*,6*S*,6a*S*)-hexahydro-2-oxo-1*H*-thieno[3,4-*d*]imidazol-6-yl)pentanoate] is shown in its enol form, reacting with a protein sulfenic acid to generate a stable, alkylated form of the protein (Poole *et al.*, 2007).

Figure 4.2.

Use of various elution conditions to recover biotinylated proteins from streptavidin beads.

A. Biotinylated AhpC (180 μ g) was incubated with 80 μ l streptavidin beads in 250 μ l total volume of 2 *M* urea in PBS, pH 7.2 and incubated for 1.5 h at 24 °C. Beads were washed 3 $\times$ 0.5 ml 2 *M* urea in PBS, pH 7.2 and aliquotted into five different tubes prior to centrifugation for 2 min at 1000 $\times$ *g* and removal of the supernatant. Elution solutions (10 μ l) were added to each tube and incubated at the appropriate temperature for 10 min. Elution conditions were: 2% SDS in 50 mM Tris-HCl, 1 mM EDTA, pH 8.0 (90 °C); 50 mM Tris-HCl, 1 mM EDTA, pH 8.0 (90 °C); 8 *M* urea with 2% CHAPS (37 °C); 8 *M* guanidine hydrochloride (GuHCl) (37 °C); and 100 mM glycine, pH 2.8 (24 °C). The amount of AhpC protein in 2 μ l of the eluted fraction was visualized by Coomassie Blue stain after SDS-PAGE. B. Sulfenic acid-containing C165S AhpC labeled with DCP-Bio1 was incubated with monoavidin beads overnight, washed three times with PBS buffer, and aliquotted into separate tubes. Samples were either incubated with 2.25 *M* ammonium hydroxide for 16 h at 24 °C or boiled in SDS sample buffer containing β -mercaptoethanol for 10 min, and AhpC in the eluant was visualized as in (A).

Figure 4.3.

Time course of sulfenic acid formation in individual proteins monitored by Western blot analysis. HEK293 cells were stimulated with TNF α for the indicated amount of time, cells were washed with PBS, and sulfenic acids were trapped by the addition of lysis buffer containing 1 mM DCP-Bio1, 10 mM *N*-ethylmaleimide, 10 mM iodoacetamide, 200 U/ml catalase, 100 mM NaCl, 100 μ M DTPA, 20 mM γ -glycerophosphate, 1 mM sodium vanadate, 50 mM sodium fluoride, 1 mM PMSF, 0.01 mg/ml aprotinin, 0.01 mg/ml leupeptin, 0.1% sodium dodecyl sulfate, 0.5% sodium deoxy-cholate, 0.5% NP-40, and 0.5% Triton-X-100 in 50 mM Tris-HCl, pH 7.5, incubated on ice for 1 h, and stored at -80°C . Biotinylated proteins were affinity captured according to the protocol described in methods, then separated by SDS-PAGE and transferred to a nitrocellulose membrane. The extent of cysteine oxidation on individual proteins was evaluated using protein-specific antibodies. Biotin-labeled AhpC was added based on protein concentrations prior to affinity capture and used as a procedural control for the biotin-based affinity capture, elution and gel loading steps. Antibodies to HSP70, PKC- β 1, and GAPDH were from Santa Cruz; antibodies to PTEN and SHP-2 were from Cell Signaling, and the AhpC antibody was purified from rabbit serum.

Figure 4.4.

SHP-2 (A) and PTEN (B) labeling is established by immunoblots to SHP-2 and PTEN after biotin affinity capture (biotin AC) and by visualizing biotinylated proteins after immunoprecipitation (IP) of SHP-2 and PTEN, but these proteins are not endogenously biotinylated. HEK293 cells were grown in DMEM media containing 10% FBS and exchanged into serum-free DMEM media for 30 min prior to the addition of the 5 mM DCP-Bio1-containing lysis buffer and incubation (see Fig. 4.3). Cell lysates were centrifuged at 14,000×g for 10 min, and the protein concentration in each supernatant was determined using the BCA protein assay. For immunoprecipitation, lysates (175 μg of protein each) were preincubated for 1 h at 4 °C with 20 μl Dynabeads-Protein A magnetic beads preequilibrated in PBS (but lacking antibody) to remove nonspecifically associating

proteins, then supernatants were transferred to tubes containing 0.5 μ l of either anti-PTEN or anti-SHP-2 antibody (Cell Signaling) and a fresh aliquot (20 μ l) of magnetic beads and incubated overnight at 4 °C. Supernatants were then removed and the beads were washed three times with PBS buffer. Immunoprecipitated proteins were eluted with 35 μ l SDS sample buffer. Affinity capture of biotinylated proteins was conducted according to the protocol described in methods. Proteins (10 μ l per sample) were separated by SDS-PAGE in 10% polyacrylamide gels, transferred to nitrocellulose membrane, and blocked with 5% milk. For immunoblotting, membranes were incubated for 2 h at 24 °C with a 1:10,000 dilution of streptavidin-HRP or for 16 h at 4 °C with a 1:1000 dilution of either anti-SHP-2 (A, Cell Signaling) or anti-PTEN antibodies (B, Cell Signaling), then visualized with Pico chemiluminescent substrate (Pierce).

Figure 4.5.

Techniques to monitor global sulfenic acid formation. (A) To visualize protein bands in samples after affinity capture of biotinylated proteins, HEK293 cells were treated (or not) with 100 nM insulin for 2 min, then sulfenic acids were trapped with 1 mM DCP-Bio1 in lysis buffer as in Fig. 4.3. Streptavidin–agarose beads were used to capture biotinylated proteins from lysates, then extensively washed with 1% SDS, 4M urea in PBS, 1 M NaCl, 100 μM ammonium bicarbonate and dH₂O. Proteins were eluted with 2% SDS in 50 mM Tris–HCl, pH 8.0, 1 mM EDTA, separated by SDS–PAGE, and stained with SYPRO Ruby (Pierce). Proteins showing increased sulfenic acid labeling in response to insulin are marked with arrows. (B) To blot for biotin after separation of total protein samples on gels, HEK293 cells were treated (or not) with TNF for the indicated amount of time, then sulfenic acids were trapped with 1 mM DCP-Bio1 in lysis buffer. Unreacted DCP-Bio1 was removed using a Bio-Gel P6 spin column and 20 μg of protein from each sample was separated on a 10% SDS–PAGE followed by transfer to nitrocellulose. Membranes were blocked overnight with 5% milk, incubated with a 1:1000 dilution of anti-biotin, HRP-conjugated antibody (Cell Signaling) for 2 h at 24 °C, and visualized with Pico chemiluminescence kit. As a loading control, 10 μg of each sample was probed for actin using an anti-actin antibody (Cell Signaling). (C) For protein analyses using 2D gels, HEK293 cells were treated with TNF or insulin and labeled with 1 mM DCP-FL1. Proteins were precipitated with cold acetone, washed with 10% TCA and 1:1 ether:ethanol, and then resolubilized in 8 M urea, 2% CHAPS, 50 mM DTT, and 0.2% ampholytes. Proteins were separated by 2D electrophoresis using pH 3–10 IPG strips (BioRad), followed by 4–20% Criterion gel (BioRad), and fluorescein labeled proteins were visualized on a Amersham STORM 840 fluorescence imager. (D) A FluoReporter biotin quantitation assay kit (Invitrogen) was used to quantify the amount of biotin incorporation into HEK293 cells before and after stimulation with TNF for 10 min. Briefly, cell lysates (~10–25 μg) were digested with *Streptomyces griseus* Protease type XIV overnight at 37 °C and diluted in PBS until each sample contained 1–2 μg total protein. After incubation for 5 min at room temperature in the

dark with an equal volume of Biotective Green reagent, fluorescence was measured on a Tecan Safire 2 fluorescence plate reader using $\lambda_{\text{ex}} = 485 \text{ nm}$ and $\lambda_{\text{em}} = 530 \text{ nm}$.

Figure 4.6.

LC/MS/MS spectrum of a DCP-Bio1-labeled tryptic peptide from PrxVI labeled on the reactive Cys residue, Cys46. HEK293 cells were grown in DMEM media to 80% confluence, serum-starved for 20 h, and treated with 100 nMTPA for 1 min. Cells were scraped, then lysed in lysis buffer containing 5 mMDCP-Bio1 and 10 mM NEM and incubated at room temperature for 30 min; proteins in the sample were further treated under denaturing conditions by adding urea to 6M and incubating for 10 min with 10 mM DTT, then adding NEM to 20 mM for another 35 min incubation. To remove excess labeling agents and protease inhibitors, the sample was exchanged into digestion buffer (2 M urea, 100 mM ammonium bicarbonate) using a G-25 spin column. Trypsin was added (1:100, w/w) and incubated at 37 °C for 15 h. Excess trypsin and undigested protein was removed by passing peptides through a 5000 molecular weight cut-off Ultrafree-MC centrifugal filter (Millipore) following digestion. For biotin affinity capture, peptides were incubated with a preequilibrated mono-avidin resin (from Pierce) at 4 °C overnight. The beads were washed several times with additional urea/bicarbonate buffer, followed by 10 mM ammonium bicarbonate, then water. Peptides were eluted with 30% acetonitrile containing 500 mM formic acid, then concentrated in a SpeedVac before analysis. Reverse phase chromatography on a C-18 column was used to resolve peptides, with a portion of the eluant injected directly into the LTQ mass spectrometer. As illustrated above, cleavage of the amide bond results in N-terminal fragments designated as “b” and C-terminal fragments designated as “y”. The masses of both sets of ions are consistent with DCP-Bio1 linked covalently to Cys46 of human peroxiredoxin VI (PrxVI) ($y_7 - y_6 = b_6 - b_5 = 498.5 \text{ m/z}$).

Table 4.1

Cysteine sulfenic acid-modified proteins identified in TNF treated HEK293 cells

Protein name	Function	Affinity capture, gel & MS ^a	Affinity capture, gel & WB	Cysteine i.d ^b
Actin ^d	Cell structure & motility	X	X	C10
Tubulin B ^d	Microtubule protein	X	X	
Heat shock protein 70 (HSP70) ^d	Chaperone	X	X	C17
PrxI ^c	Peroxidase		X	
PrxII ^c	Peroxidase	X	X	
PrxIII ^c	Peroxidase		X	
PrxIV ^c	Peroxidase		X	
-Enolase ^d	Glycolytic enzyme		X	
Glyceraldehyde-3-P dehydrogenase (GAPDH) ^c	Glycolytic enzyme	X	X	C151/155
Protein kinase C (PKC- 1) ^d	Signaling kinase		X	
Protein and lipid phosphatase (PTEN) ^d	Signaling phosphatase		X	
SH2 homology protein 2 (SHP-2) ^c	Signaling phosphatase		X	

^a Gels were stained with Coomassie Blue dye, and protein spots of interest were excised, washed three times with 50% acetonitrile, dried, washed twice, successively, with 200 μ l ammonium bicarbonate and 100% acetonitrile, dried, and digested with 0.1 mg/ml trypsin in 50 mM ammonium bicarbonate for 16 h at 37 °C. Peptides in the supernatant were combined with peptides eluted with 50% acetonitrile and spotted using a matrix containing 30 mg of dihydroxybenzoic acid in 1 ml 50% acetonitrile, 0.1% trifluoroacetic acid. Protein identifications were made using a Bruker Autoflex MALDI-TOF mass spectrometer with dihydroxybenzoic acid as the matrix, and data were analyzed using MASCOT software (www.matrixscience.com).

^b In these cases, identification of the labeled peptide by mass spectrometry gave an unambiguous determination of the labeled Cys residue, except for GAPDH, which has two Cys residues on the MALDI-TOF-identified labeled peptide; based on data from other laboratories, Cys151 has been shown to form a sulfenic acid.

^c Data present in literature showing sulfenic acid formation.

^d Redox regulation known, but sulfenic acid not previously identified.