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Methods for the determination and quantification of the reactive thiol proteome

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ABSTRACT

Protein thiol modifications occur under both physiological and pathological conditions and have been shown to contribute to changes in protein structure, function, and redox signaling. The majority of protein thiol modifications occur on cysteine residues that have a low pK_a; these nucleophilic proteins comprise the "reactive thiol proteome." The most reactive members of this proteome are typically low-abundance proteins. Therefore, sensitive and quantitative methods are needed to detect and measure thiol modifications in biological samples. To accomplish this, we have standardized the usage of biotinylated and fluorophore-labeled alkylating agents, such as biotinylated iodoacetamide (IAM) and *N*-ethylmaleimide (NEM) and BODIPY-labeled IAM and NEM, for use in one- and two-dimensional proteomic strategies. Purified fractions of cytochrome *c* and glyceraldehyde-3-phosphate dehydrogenase were conjugated to a known amount of biotin or BODIPY fluorophore to create an external standard that can be run on standard SDS–PAGE gels, which allows for the quantification of protein thiols from biological samples by Western blotting or fluorescence imaging. A detailed protocol is provided for using thiol-reactive probes and making external standards for visualizing and measuring protein thiol modifications in biological samples.

Cysteinyl protein thiols play crucial roles in enzyme catalysis, protein structure, maintenance of the cellular redox potential, and cell signaling [1]. The properties that make cysteine ideal for these redox-based reactions, however, also make it exceptionally vulnerable to oxidation by reactive oxygen or nitrogen species (ROS/RNS) or to modification by environmental or endogenous electrophiles [1,2]. The average pK_a of the cysteine residue thiol is ~8.5, which at cytosolic physiological pH is less likely to react with ROS/RNS or electrophiles. However, many proteins have domains that result in a substantial lowering of the pK_a thiol group, such that they are predominantly in the reactive thiolate anion form at physiological pH. It is also important to note that the differences in the local intracellular environment (e.g., pH and hydrophobicity) will also have an impact on protein thiol reactivity. For example, the intramitochondrial pH is typically more alkaline than that of the cytosol, which will probably affect the composition of thiol-reactive proteins [3,4]. These proteins collectively make up the "reactive thiol proteome."

Several methods have been used to detect and measure thiol modifications. These include direct detection techniques for individual modifications (e.g., protein modifications induced by nitric oxide, glutathione, or electrophilic lipids) and strategies for the detection and quantification of overall cellular thiol modification [1,2]. Detection of specific thiol modifications utilizes either antibody-based detection approaches or chemical approaches that facilitate tagging of the modified thiol group, whereas detection of the reactive thiol proteome is predominantly based on tagging methodologies that employ thiol-reactive probes. Both of these approaches have advantages and disadvantages. The obvious advantage of probing for individual modifications is that specific modifications can be monitored and associated with pathological or physiological mechanisms; however, the drawback with such an approach is that other modifications to the thiol proteome that may occur simultaneously may be overlooked. In this respect, the development of external standards is particularly important because it allows for the quantification of protein thiol modifications. This information can be valuable in assessing the biological impact of a modification. For example, if only 1 protein molecule in 100 molecules is modified, then it is unlikely that this modification will have a significant biological impact. Tagging the unmodified or reduced thiol pool allows for a broad view of the redox state of the cell or tissue and can be used to identify oxidatively modified proteins. However, this approach does not distinguish thiols that are specifically S-nitrosated, S-glutathiolated, oxidized, or modified by electrophilic lipids. Several excellent reviews and articles on the detection of specific modifications and approaches for tagging the unmodified protein thiol pool are available [1,2,5–10]. The purpose of this article is to provide a detailed protocol for the detection and quantification of the reactive thiol proteome using biotin- and fluorescence-based proteomic approaches.

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Principles

Alkylation of free thiols and detection of alkylated proteins is an effective strategy for evaluating the reactive thiol proteome. *N*-ethylmaleimide (NEM), iodoacetamide (IAM), and iodoacetic acid (IAA) are commonly used for protein thiol alkylation. Additionally, radiolabeled, biotin-conjugated, and fluorophore-labeled forms of these and similar compounds are commercially available. Nevertheless, the decision of which compound to use experimentally should be based on the suitability of the thiol alkylation chemistry and the detection method employed. Moreover, the usage of IAA may or may not be desirable for proteomic strategies (depending on the experimental question being addressed) because it carries with it a negative charge that may shift the isoelectric point of labeled proteins.

The underlying chemistries of sulfhydryl modification by thiol-alkylating agents are distinct and confer differences in their reactions with proteins. IAM and IAA yield carbamidomethylated and carboxymethylated cysteines, respectively, by bimolecular nucleophilic substitution (S_N2) reactions [11,12]. The lone pair of electrons in the deprotonated thiol (thiolate anion; S^-) act as the nucleophile and attack the electron-deficient electrophilic center of IAM/IAA, expelling iodine anion as the leaving group (Fig. 1A). This reaction is second order, with the rate of reaction depending on the nucleophile concentration (S^-), the concentration of the substrate itself (IAA/IAM), and the pH and proticity of the solvent. The reaction of NEM with thiols is based on a Michael-type addition reaction [11], in which the thiolate anion attacks the electrophilic center of the C = C bond of the maleimide group to form a thioether bond between the thiol and the maleimide (Fig. 1B). The reaction of NEM with thiols is faster than that of IAM or IAA and less dependent on pH [11,12]. However, NEM may be less specific than iodo derivatives; at alkaline pH, NEM also reacts with the side chains of lysine and histidine [2]. The comparative effectiveness of protein thiol alkylation between NEM, IAM, and IAA was demonstrated in a study by Rogers et al. [12].

Biotin-based tagging techniques have been used to monitor the intracellular thiol status of proteins after exposure to ROS/RNS or electrophilic compounds [10,13–17]. To assess protein thiol modification in general, thiols can be labeled directly with biotin-tagged reagents such as biotinylated IAM (BIAM) or biotinylated NEM (Bt-NEM), and the biotin signal can subsequently be measured by standard immunoblotting-type protocols using streptavidin-conjugated horseradish peroxidase (HRP). In this case, the loss of the biotin signal is proportional to the degree of thiol modification. To quantify protein thiol content, we have developed biotinylated standard proteins containing known amounts of biotin per mole of protein [10].

The major advantages of this biotin–thiol tagging technique include: (1) the extremely high affinity of avidin and streptavidin for biotin ($K_d \approx 10^{-15}$ M) [18,19]; (2) the binding of streptavidin, which, unlike an antibody, is not readily affected by flanking residues at the site of protein modification; (3) affinity resins being available for purification of the biotinylated proteins; and (4) the biotin tag being easily and accurately quantified using biotinylated standards. Nevertheless, there are some drawbacks to using biotin as a tag, which must also be considered. For example, in contrast to antibody-based methods, the high affinity of streptavidin for biotin results in an association that is practically (though not formally) irreversible. The technical implications are that Western blots using enzyme-conjugated avidin/streptavidin as a means of detection cannot be stripped after development. In addition, some proteins such as carboxylases have biotin covalently attached as an enzymatic cofactor, and these proteins can give false positive results that must be accounted for during data interpretation and quantification. Last, the conditions for transfer of the proteins from the SDS–PAGE gel to PVDF or nitrocellulose membranes must be standardized to ensure that all of the protein is transferred. In many cases, proteins are not thoroughly transferred from the gel, and proteins of different molecular weights tend to transfer at different rates. This could lead to inaccurate estimates of the amount of biotin incorporated based on the initial protein loaded for SDS–PAGE.

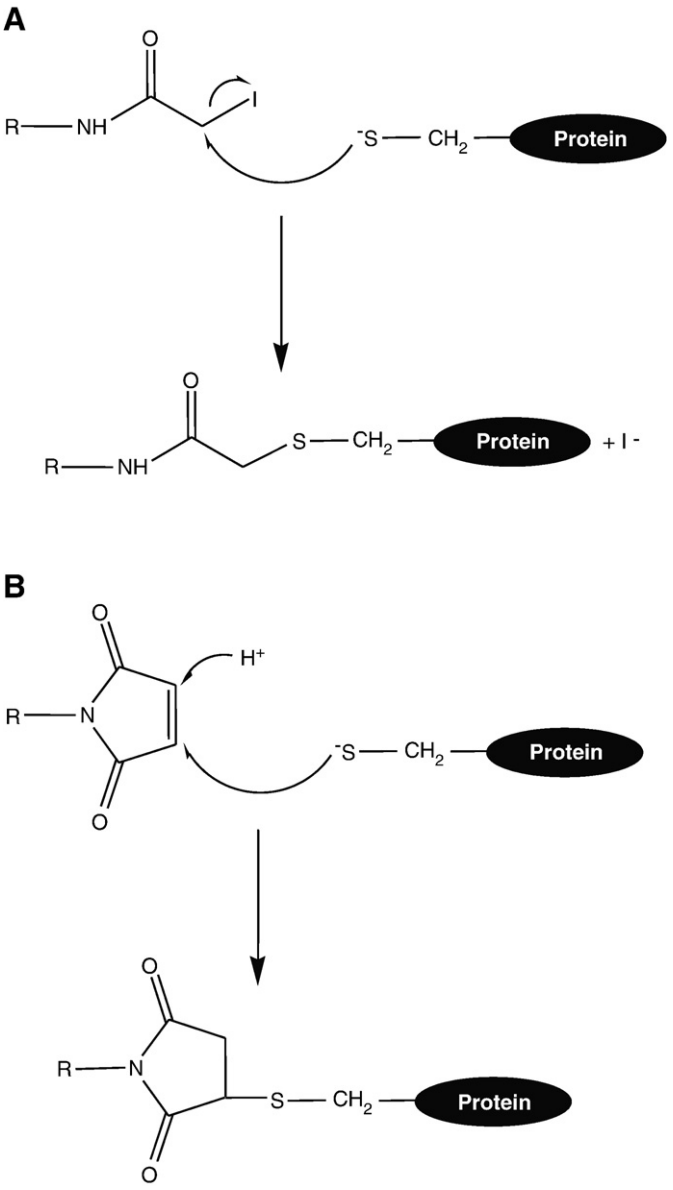

Fig. 1. Reactions of labeled protein-alkylating agents with protein cysteinyl residues. This protocol describes the use of iodoacetamide- and *N*-ethylmaleimide-based probes for the identification and quantification of protein thiol modification. (A) Reaction of an iodoacetamide probe with a reactive thiol. The thiolate anion of cysteine reacts with iodoacetamide in a nucleophilic displacement (S_N2) reaction in which iodine is the leaving group. This forms an irreversible thioether bond between the probe and the protein. (B) Reaction of an *N*-ethylmaleimide probe with a reactive thiol. In this reaction, the thiolate anion of cysteine forms a thioether bond with *N*-ethylmaleimide through an addition reaction. The R groups in (A) and (B) represent the biotin or fluorophore tags used in this protocol.

Fluorophore-tagged alkylating agents are also an option for use in the assessment of the reactive thiol proteome [9,20]. A major advantage to using fluorophore-tagged alkylating agents is that the transfer step can be omitted because Western blotting is not required. After alkylation and separation by one-dimensional (1D) SDS–PAGE or by 2D proteomics techniques, the level of alkylation can be measured in-gel using detection techniques based on the emission/excitation characteristics of the particular fluorophore. False positives that occur with biotin-tagging techniques (due to endogenous biotin-containing enzymes) are also circumvented using fluorescence-based tagging

techniques. Generally, fluorescence-based approaches such as these are highly sensitive and have a large dynamic range. However, one disadvantage is that some fluorophores are light sensitive, and consequently, much of the work must be done under low-light conditions. Also, unless an antibody to the fluorophore itself exists, pull-down assays cannot be performed using fluorophore-labeled compounds. In this methods article, we also describe a BODIPY-labeled standard that can be used for quantifying the reactive thiol proteome.

Materials

- *N*-(Biotinoyl)-*N*′-(iodoacetyl)ethylenediamine (BIAM) (Invitrogen, Carlsbad, CA, USA, Product B1591)
- Maleimide–PEO_2–biotin (Bt-NEM) (Thermo Scientific, Rockford, IL, USA, Product 21902)
- *N*-(4,4-Difluoro-5,7-dimethyl-4-bora-3*a*,4*a*-diaza-*s*-indacene-3-yl) methyl iodoacetamide (BODIPY-IAM) (Invitrogen, Product D6003)
- BODIPY-FL–*N*-(2-aminoethyl)maleimide (BODIPY-NEM) (Invitrogen, Product B10250)
- BODIPY-FL-SSE (sulfosuccinimidyl ester) (Invitrogen, Product D6140)
- Complete Mini protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN, USA, Product 11 836 153 001)
- 1-Ethyl-1*H*-pyrrole-2,5-dione (NEM) (Fisher Scientific, Pittsburgh, PA, USA, Product O2829-25)
- β-Mercaptoethanol (Sigma–Aldrich, St. Louis, MO, USA, Product M7154)
- Lowry DC protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA, Product 500-0116)
- SuperSignal West chemiluminescent substrate (Pierce Biotechnology, Rockford, IL, USA, Product 34077)
- ECL Plus Western blotting detection reagents (GE Healthcare, Piscataway, NJ, USA, Product RPN2132)
- Hybond low-fluorescence PVDF membrane (GE Healthcare, Product RPN303LFP)
- Biological samples such as whole homogenate tissue fractions, isolated mitochondria, etc.
- Labeling buffers
  - Low pH buffer (pH 6.5): 50 mM 2-(*N*-morpholino)ethanesulfonic acid, pH 6.5, containing 1% Triton X-100 (v/v) and 100 mM NaCl. Add one tablet Complete Mini protease inhibitor cocktail to 10 ml of buffer immediately before using. *Note:* omit NaCl if samples will be used for isoelectric focusing.
  - High pH buffer (pH 8.5): 10 mM Tris–HCl, pH 8.5, containing 1% Triton X-100 (v/v) and 100 mM NaCl. Add one tablet Complete Mini protease inhibitor cocktail to 10 ml of buffer immediately before using. *Note:* omit NaCl if samples will be used for isoelectric focusing.
  - Neutral pH buffer (pH 7.0): 25 mM Hepes, 100 mM NaCl, 1% NP-40 (v/v), 0.1% SDS, and 1 mM EDTA. Add one tablet Complete Mini protease inhibitor cocktail to 10 ml of buffer immediately before using. *Note:* omit NaCl and SDS if using for isoelectric focusing.
- Horse heart cytochrome *c* (Sigma–Aldrich, Product C2867)
- Glyceraldehyde-3-phosphate dehydrogenase from rabbit muscle (Sigma–Aldrich, Product 068K7405)
- Sulfo-NHS-LC-biotin (Pierce Biotechnology, Product 127062-22-0)
- PD-10 gel filtration column (GE Healthcare, Product 17-0851-01)
- Slide-A-Lyzer 10K, dialysis cassette, 10,000 MW (Pierce Biotechnology, Product 66425)
- Zeba desalt spin columns (Thermo Scientific, Product 89889)
- 2-(4′-Hydroxyazobenzene) benzoic acid (HABA) reagent (Pierce Biotechnology, Product 28010)
- ImmunoPure avidin (Pierce Biotechnology, Product 21121)
- Sodium dithionite (Sigma–Aldrich, Product 28-2925)
- Sypro Ruby protein gel stain (Invitrogen, Product S12000)
- Streptavidin, horseradish peroxidase conjugated (streptavidin–HRP) (GE Healthcare, Product RPN1231)
- Blotting-grade milk (Bio-Rad Laboratories, Product 170-6404)
- Tris-buffered saline containing 0.05–0.1% Tween 20 (TBS-T) (phosphate-buffered saline (PBS) can be used interchangeably with TBS)

Instrumentation

- UV/Vis spectrophotometer
- Mini-Protean electrophoresis system (Bio-Rad)
- Protean isoelectric focusing cell with accompanying materials and reagents (Bio-Rad)
- CCD camera imaging system such as the FluorChem 8000 (for chemiluminescence imaging; AlphaInnotech, San Leandro, CA, USA) or a Typhoon variable mode imager (Model 9400 or Trio for both fluorescence and chemifluorescence imaging; GE Healthcare)

Protocol

Preparation and calibration of biotinylated cytochrome c

We have standardized the conjugation of biotin to cytochrome *c* for quantifying biotin in Western blotting procedures [10]. This conjugation reaction utilizes an amine-reactive succinimidyl ester that will react with lysine residues to form a stable amide bond.

(1) Prepare a stock solution of horse heart cytochrome *c* (cyt *c;* MW 12.4 kDa) by dissolving 10 mg in 1 ml of PBS. In addition to cyt *c*, proteins of various molecular weights, including horse heart myoglobin (MW 17 kDa) and soybean trypsin inhibitor (MW 21.5 kDa), can be coupled with biotin as described for cyt *c*. Bovine serum albumin is not recommended, because the quantitative relationship between biotin content and fluorescent signal is not consistent (unpublished observations). This is probably due to a direct interaction of HABA, which is used to quantify protein biotinylation, with albumin [21].
(2) Prepare the biotin derivatizing reagent—sulfo-NHS-LC-biotin—by dissolving 5.5 mg of biotin reagent in 1 ml of PBS.
(3) Combine 1 ml of protein solution (10 mg cyt c/ml PBS) and 1 ml of biotin solution (5.5 mg/ml). Allow the reaction to proceed for 4 h on ice. In our experience, the optimal molar ratio of biotin:protein is 10:1, which in the case of cyt c resulted in the addition of ~4.6 mol of biotin per mole of protein.
(4) Remove unreacted biotin by gel filtration using a PD-10 column. The column should be preequilibrated with 25 ml of PBS. Allow the PBS to drain to the level of the column bed, add the biotinylated protein solution (maximum volume 2 ml), and again allow the solution to drain to the level of the column bed. Add 30 ml of PBS into the column to elute the protein sample. Note: with cyt c and other colored proteins, the protein can be monitored visually as it passes through the column. As the band elutes from the column, begin collecting 1-ml fractions. For uncolored proteins, collect 30–40 0.5-ml fractions (4–5 drops) and screen for protein in each fraction by spectrophotometry at 280 nm or protein assay. To minimize free biotin contamination, collect the first 1–3 tubes from the first peak that contains protein.
(5) Determine cyt c concentration and biotin incorporation as described below.

Measurement of protein concentration

The cyt *c* concentration is most accurately measured using the spectral properties of the covalently bound heme prosthetic group in its reduced state. For other proteins, we use the Lowry protein assay method to determine protein concentration [22]. The amount of biotin

(moles) for a given amount of protein (moles) can be calculated from the concentrations of biotin (determined below by the HABA assay) and protein. We have also confirmed these calculations with detailed mass spectrometric analysis [10].

(1) Add 1 ml of PBS to a cuvette and blank the spectrophotometer using a wavelength scan from 500 to 600 nm.
(2) Add a measured aliquot of biotinylated cyt *c* into the PBS solution and add trace sodium dithionite (typically, a few grains of fresh powder) to reduce the heme group. Measure the absorbance using a wavelength scan from 500 to 600 nm. As shown in Fig. 2A, cyt *c* maximally absorbs at 550 nm. Calculate the biotinylated cyt *c* concentration taking into account the dilution factor of the measured aliquot using the absorbance at 550 nm with an extinction coefficient of 27,600 M^{-1} cm^{-1}.

Determination of biotin incorporation in standard proteins (HABA assay)

Biotin incorporation is determined using a colorimetric HABA dye displacement assay. In this assay, the colored dye HABA reversibly binds avidin and is displaced by biotin, resulting in a decrease in the absorbance of HABA at 500 nm [23].

(1) Prepare a stock solution of HABA by dissolving 24.2 mg of HABA in 10 ml of 20 mM NaOH (10 mM final HABA concentration). This solution may be stored at 4 °C for several weeks.
(2) Prepare a stock solution of avidin by dissolving 10 mg of ImmunoPure avidin in 19.4 ml of PBS.
(3) Add 0.6 ml of 10 mM HABA stock solution to the solution from step 2 to make a HABA–avidin solution.

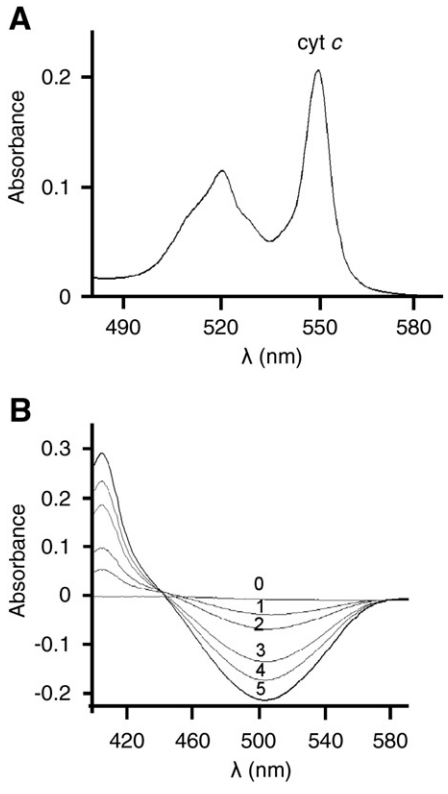

Fig. 2. Measurement of biotin incorporation into cytochrome *c*. After incubation of cyt *c* with sulfo-NHS-LC-biotin, biotin incorporation is determined by the colorimetric HABA dye displacement assay. (A) Measurement of cyt *c* concentration. Cyt *c* is best measured spectrophotometrically in its reduced state. As shown after dithionite treatment, cyt *c* absorbs maximally at 550 nm. (B) HABA assay. The colored dye HABA binds avidin and is displaced by biotin, resulting in a decrease in the absorbance of HABA at 500 nm. The decrease in absorbance due to the displacement of the HABA dye in 1–5 μg of biotinylated cyt *c* is shown.

(4) Combine 0.5 ml of HABA–avidin solution with 0.5 ml of PBS in a 1-ml cuvette and blank the spectrophotometer with this solution using a wavelength scan from 300 to 700 nm. Add the biotinylated cyt *c* into the blanked HABA–avidin solution and measure absorbance again as described above. As shown in Fig. 2B, increasing the amount of biotinylated protein results in dissociation of HABA from avidin, resulting in a decrease in absorbance at 500 nm.

We have observed that the linear range of this assay, in which an increase in biotin is represented by a proportional decrease in absorbance, is somewhat narrow [10]. Therefore, it is necessary to repeat measurements using varying amounts of biotinylated protein, usually 1–20 μg. At this point, the absorbance at 500 nm may be plotted as a function of the protein amount added (in μg). In the linear range of the plot, quantitate the biotin incorporation in solution according to the extinction coefficient 34,000 M^{-1} cm^{-1}. In the case of colored proteins, it may be necessary to correct for the absorbance of the protein itself by preparing a solution of protein in PBS that does not contain HABA–avidin. This absorbance should be subtracted from that of the HABA–avidin solution containing the protein.

Preparation of BODIPY-labeled glyceraldehyde-3-phosphate dehydrogenase (BD-GAPDH) standard

We have also standardized the conjugation and usage of BODIPY for the quantification of protein thiols using BODIPY-IAM. Similar to biotinylated cyt *c*, the BODIPY conjugation reaction utilizes an amine-reactive succinimidyl ester that will react with lysine residues to form a stable amide bond.

(1) Make a 2 mg/ml stock of GAPDH (MW 37 kDa) in PBS (pH 7.4).
(2) Make a ~30 mM stock solution of BODIPY-FL-SSE in dimethyl sulfoxide (DMSO) based on the formula weight. Determine the actual concentration of the BODIPY-FL-SSE spectrophotometrically. To do this, make a 1:10 dilution of the stock BODIPY-FL-SSE in DMSO. Then, add 999 μl of methanol to a quartz cuvette and blank the instrument. Add 1 μl of the 1:10 BODIPY-FL-SSE stock to the cuvette, mix by gentle pipetting, and perform a wavelength scan from 250 to 700 nm. As shown in Fig. 3A, BODIPY absorbance is maximal at 502 nm.
(3) Calculate the stock concentration of BODIPY-FL-SSE using $\varepsilon = 76{,}000$ M^{-1} cm^{-1}, accounting for both dilutions (e.g., 10,000× dilution).
(4) Add 54 nmol of BODIPY-FL-SSE to 1 ml of the 2 mg/ml (54 μM) GAPDH solution. This will give a 1:1 molar ratio of BODIPY-FL-SSE:GAPDH.
(5) Incubate the mixture for 4 h on ice.
(6) Dialyze the reaction mixture against 500 ml of PBS, pH 7.4, in a 10,000 MW-cutoff dialysis cassette for 1 h at 4 °C. Change the buffer and dialyze again overnight. Change the dialysis buffer once more the following morning and dialyze further for 1 h.
(7) Run the dialyzed sample through a Zeba desalting column at 1000 *g* for 2 min.
(8) Analyze the BD-GAPDH spectrophotometrically in a quartz cuvette. Set the wavelength scan to 250–700 nm. Blank the instrument against PBS and add the BD-GAPDH to the cuvette. As shown in Fig. 3B, there is a slight red shift in the BODIPY absorbance spectrum; the maximal absorbance of BD-GAPDH occurs at 506 nm. Calculate the BD concentration using $\varepsilon = 76{,}000$ M^{-1} cm^{-1}, accounting for dilution into the cuvette.
(9) Determine the protein concentration of the BD-GAPDH by the Lowry method. A rough estimate is given by the absorbance at 280 nm, shown in the wavelength scan in Fig. 3B.

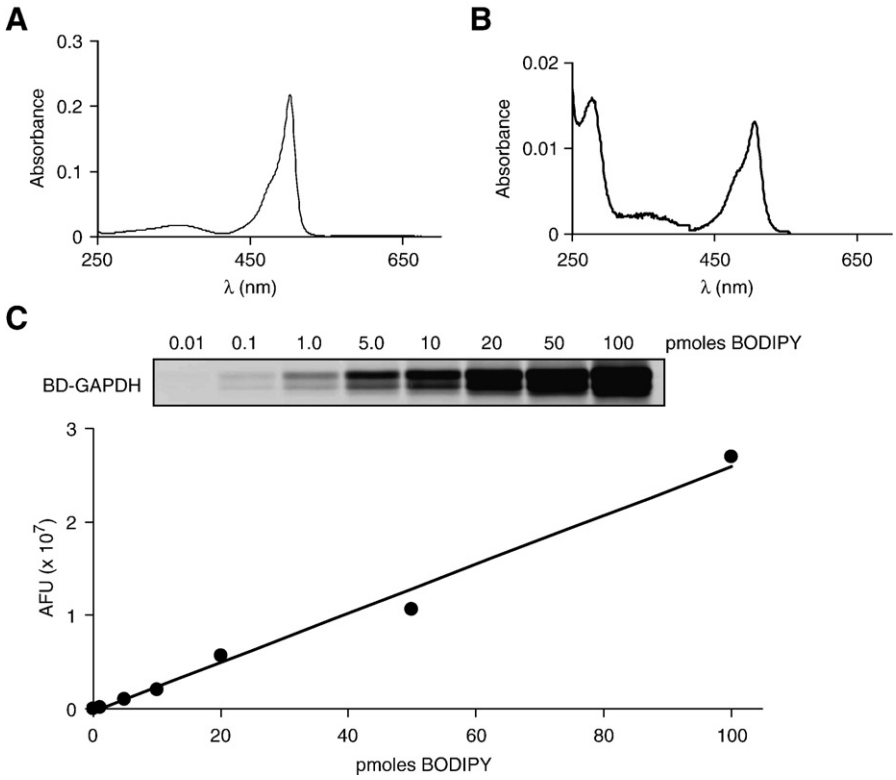

Fig. 3. Measurement and fluorescence characteristics of BODIPY-labeled glyceraldehyde-3-phosphate dehydrogenase (BD-GAPDH). (A) Spectral characteristics of BODIPY-FL–iodoacetamide. (B) Spectral characteristics of BD-GAPDH. (C) Sensitivity and dynamic range of BD-GAPDH. BD-GAPDH (0.01–100 pmol BODIPY) was resolved by 12% SDS–PAGE and imaged using a Typhoon Trio imager. The picomoles of BODIPY were plotted against the arbitrary fluorescence units (AFU) to generate a standard curve. $y = 3 \times 10^6 x$, $R^2 = 0.99$.

(10) Calculate the moles of BODIPY per milligram of protein by dividing the concentration of BODIPY in the prepared sample by the protein concentration (from steps 8 and 9).
(11) To prepare the standard for SDS-PAGE, add the BD-GAPDH to standard SDS–PAGE loading buffer containing 5% β-mercaptoethanol or 30 mM dithiothreitol. BD-GAPDH is best resolved on 10–12% SDS–polyacrylamide gels. As shown in Fig. 3C, the BODIPY standard typically has a dynamic range of ~10 fmol to 100 pmol.

Note: bromophenol blue can quench the fluorescence of BODIPY. It is advised that dyes are omitted from the SDS sample loading buffer in this protocol. A 5× bromophenol blue-free loading buffer can be prepared using 62.5 mM Tris, pH 6.8, containing 8% SDS and 30% glycerol.

"Snap-shot" labeling of free protein thiols in biological samples using BIAM, Bt-NEM, BODIPY-IAM, or BODIPY-NEM

The BIAM- or BODIPY-labeled alkylating agents can be used at the time of cell lysis or tissue homogenization to evaluate the thiol redox state. This gives a snap-shot of the thiol status at one point in time. Below is a general protocol for snap-shot thiol labeling:

(1) Biological samples should be prepared in the high pH, low pH, or neutral pH buffer and centrifuged to remove cell and tissue debris. The protein concentration should be at least 1 mg/ml. The neutral pH buffer is a convenient buffer to use because it is similar to the standard RIPA buffer that is used for immunoprecipitation and is compatible with most standard immunoprecipitation protocols. Note that there will probably be more extensive labeling with this buffer versus the low pH (pH 6.5) buffer. After lysis or homogenization, keep the samples on ice until step 6.
(2) Measure the protein concentration in the biological samples using the Lowry DC assay or another assay that is compatible with the levels of detergent found in the labeling buffers.
(3) Normalize each sample to 1–5 mg/ml protein concentration by using the appropriate labeling buffer as the diluent. Because the reaction of IAM with thiols is a bimolecular reaction (i.e., the reaction rate is dependent on both reactant (IAM) and substrate (S^-)), it is important that all samples be of equal protein concentrations.
(4) Prepare a 500 mM stock solution of β-mercaptoethanol in water. The concentration of undiluted β-mercaptoethanol is 14.3 M. This will be used to stop the alkylation reaction with protein thiols. Alternatively, excess unlabeled NEM can be used to block remaining thiols if reducing agents are not desired. For this, make a 100 mM stock of unlabeled NEM in labeling buffer. Keep on ice until use.
(5) Prepare a 10 mM stock of BIAM, Bt-NEM, BODIPY-IAM, or BODIPY-NEM by dissolving in dimethylformamide or DMSO. These solutions should be prepared *immediately* before use, protected from light, and kept at room temperature.
(6) Add 1 μl of BIAM, Bt-NEM, BODIPY-IAM, or BODIPY-NEM alkylating agent to 200 μl of sample homogenate or lysate. The final concentration of labeled alkylating agent will be 50 μM. If more or less labeling is desired, the amount of stock alkylating agent added to the sample can be adjusted to make a 25–250 μM final concentration. However, in our experience 50 μM provides adequate labeling.
(7) Incubate the samples for 30 min in the dark at room temperature.
(8) Stop the reaction by adding 10 μl of 500 mM β-mercaptoethanol to each sample (final concentration ~20 mM). Alternatively, add 50 μl of 100 mM unlabeled NEM to the samples (final concentration 20 mM) to block the remaining reduced thiols. Vortex the samples and place on ice. Note the changes in protein concentration owing to the addition of the reagents in steps 6 and 8.
(9) Load 5–20 μg of protein per well and include preferably three concentrations of biotinylated cyt *c* or BD-GAPDH (e.g., 1, 5, and

20 pmol of biotin or BODIPY in additional wells of the gel). After electrophoresis, analyze protein labeling by densitometry (see Analysis, under Notes on protein separation, blotting, and image analysis using thiol labeling agents, for details). If using BODIPY-labeled IAM or NEM, do not remove the gel from the glass plates after electrophoresis. The image can be conveniently developed with the gel in the glass plates on a Typhoon Imager (see In-gel fluorescence imaging, under Notes on protein separation, blotting, and image analysis using thiol labeling agents, for imaging details). After imaging, the gel can be stained (e.g., Coomassie or Sypro Ruby stain) or proteins in the gel can be transferred to PVDF or nitrocellulose membranes for other blotting applications. For detection of the biotin label, transfer the proteins to PVDF or nitrocellulose membranes and probe with streptavidin–HRP.

As shown in Figs. 4A–4C, BIAM will alkylate fewer thiols than Bt-NEM under these conditions. This is due to the more reactive nature of maleimide compared to iodoacetamide. If probing the reactive thiol proteome, it may be advisable to use BIAM. This will ensure that only the most reactive cysteines will be labeled, which could give more insight into the protein targets affected during conditions such as oxidative stress. The buffers used in these protocols are also compatible with 2D electrophoresis. As shown in Figs. 4D and 4E, BIAM-labeled proteins resolve well on isoelectric focusing SDS–PAGE gels.

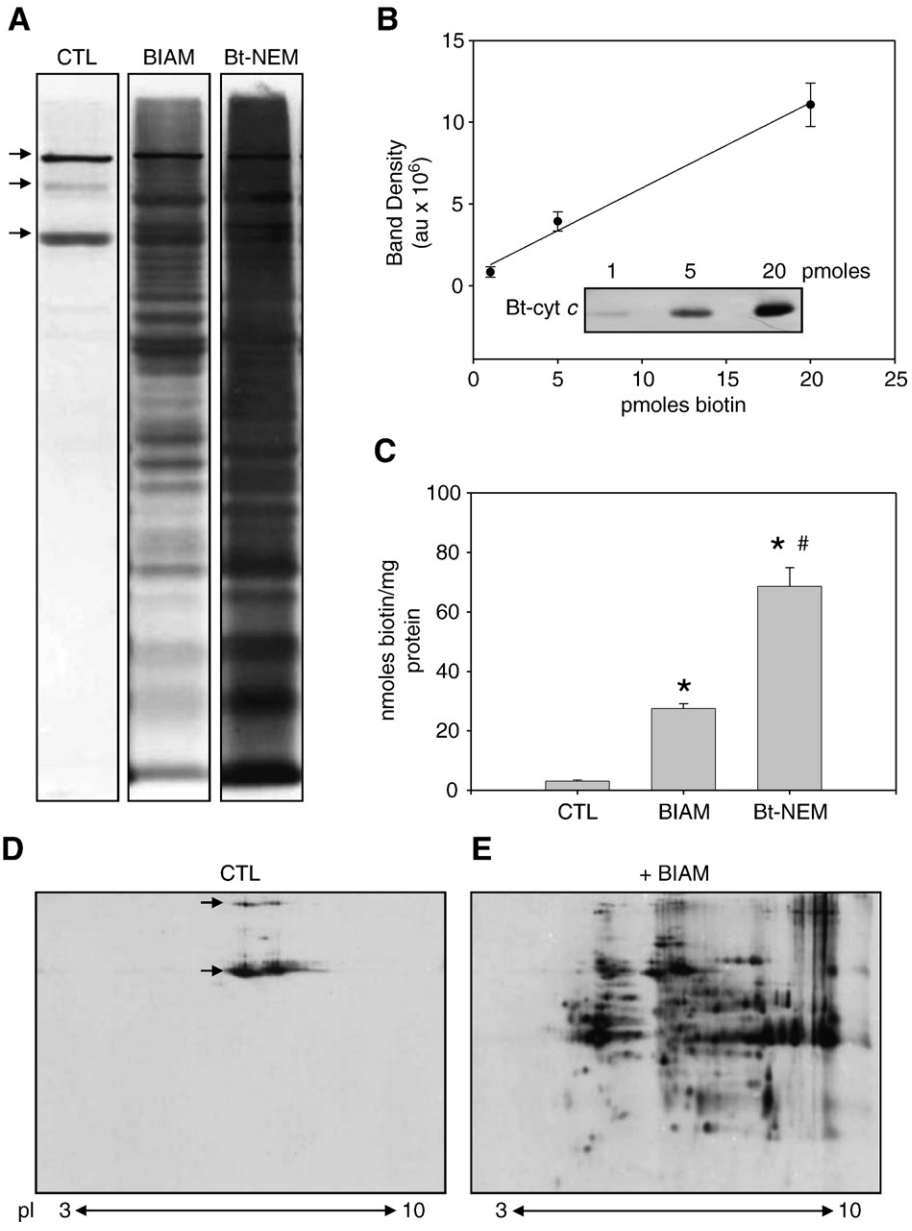

Fig. 4. Differences in thiol labeling between BIAM and Bt-NEM. (A) Western blots of biotinylated proteins. Mitochondrial proteins were treated with vehicle (DMSO; CTL), 150 μM BIAM, or 150 μM Bt-NEM under identical conditions. After alkylation with BIAM or Bt-NEM, 5 μg of protein was resolved by 12% SDS–PAGE and transferred to Hybond low-fluorescence PVDF membranes. The blots were probed with streptavidin–HRP, developed with ECL Plus reagents, and imaged using a Typhoon variable mode imager. The arrows at the far left indicate endogenous biotin-containing proteins. (B) Standard curve of streptavidin–HRP signal using biotinylated cytochrome *c* (Bt-cyt *c*) as a standard that was run on the same gels as shown in (A). au, arbitrary units; $y = 5.22 \times 10^5 x$, $R^2 = 0.99$. (C) Quantification of the amount of biotinylated protein from the blots in (A) using the standard curve in (B). $n = 3$/group; $^*p < 0.0002$ vs CTL, $\#p < 0.005$ vs BIAM. (D) Proteins (15 μg) in untreated mitochondrial lysates were focused using pH 3–10 nonlinear IPG strips and resolved by 12% SDS–PAGE in the second dimension. The proteins were then transferred to PVDF membranes, incubated with streptavidin–HRP, developed, and imaged using a Typhoon imager. Shown by the arrows are the endogenous biotin-containing carboxylases present in the mitochondria. (E) 2D profile of BIAM-modified proteins from mitochondria treated with BIAM as described for (A) (+BIAM; 15 μg protein).

Note: although fluorescence, chemiluminescence, and chemifluorescence techniques work well, the quality of the results will be dictated by the imaging system available and the level of alkylation achieved during the reaction. For the best possible results, the protocol should be optimized in each laboratory and with each imaging system. In our experience, the best resolution is achieved using BODIPY-labeled alkylating agents on a Typhoon imager; biotin-labeled alkylating agents also work well in chemifluorescence systems. Chemiluminescence systems work well, but may have less resolution than the above two methods. This is shown in Fig. 5, in which identical samples were derivatized with BODIPY-IAM or BIAM; the images show greater resolution of samples derivatized with BODIPY-IAM and imaged by in-gel fluorescence compared to BIAM-derivatized gels imaged by chemiluminescence methods.

In situ thiol labeling of biological samples

We have found that BODIPY-IAM is also useful for labeling thiols in situ in cell culture. This would be more desirable compared to snap-shot labeling if the experimenter wishes to understand how redox status changes temporally under a given set of conditions. An example of how this could be used in cell culture is shown in Fig. 6. Mesangial cells were treated with 0 or 1 mM diamide for 10 min. Cells were then treated with 50 μM BODIPY-IAM (added directly into the medium) for 30 min, followed by cell lysis in buffer containing 1 mM dithiothreitol (DTT). As described in step 8 under "Snap-shot" labeling of free protein thiols, excess alkylating agents such as NEM can be used instead of DTT to prevent further alkylation reactions from occurring after cell lysis. After cell harvest, proteins in the lysates were resolved by SDS–PAGE and imaged in-gel using a Typhoon Trio imager. As shown in Figs. 6A–6C, cells treated with diamide were labeled less extensively (i.e., -5.1 ± 0.9 nmol BODIPY/mg protein) than untreated cells, which is probably due to increased formation of protein-mixed disulfides. Because the ratio of reaction of iodoacetamide with thiols is 1:1, this suggests that the diamide treatment led to the oxidation of ~5 nmol thiol/mg protein. When separated by 2D electrophoresis, diamide was shown to oxidize a large number of proteins (Figs. 6D and 6E), which could be identified by mass spectrometry.

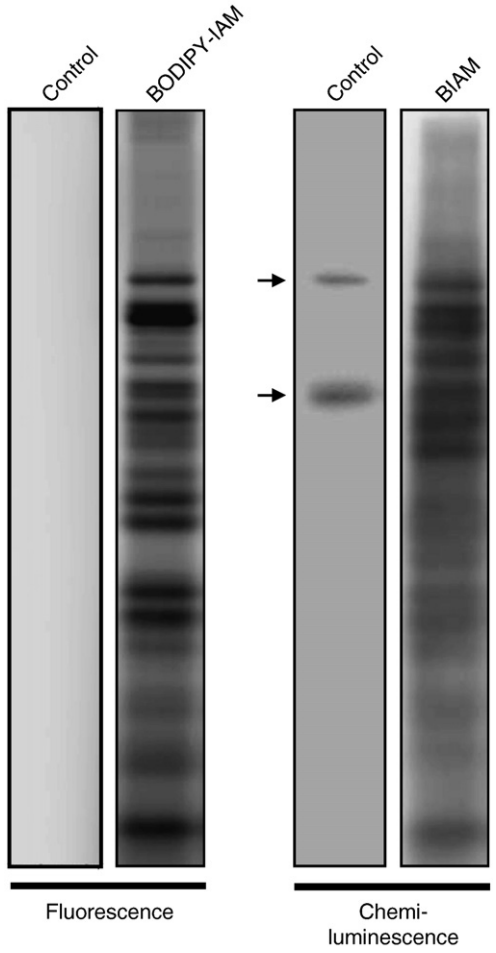

Fig. 5. Assessment of protein thiol modification using fluorophore-labeled iodoacetamide and biotinylated iodoacetamide (BIAM). Isolated rat kidney mitochondria were treated identically with 100 μM BODIPY-iodoacetamide (BODIPY-IAM) or BIAM. The reaction was then quenched by the addition of excess reducing agent (dithiothreitol). (Left) Equal amounts of protein from samples treated with BODIPY-IAM were separated by SDS–PAGE, and the images were developed on the Typhoon imaging system. (Right) To compare the fluorescent image with a chemiluminescent image, proteins from an identical sample treated with BIAM were transferred to PVDF membranes, probed with streptavidin–HRP, and imaged using a CCD camera imager.

Notes on protein separation, blotting, and image analysis using thiol labeling agents

A key aspect for accurate quantitation of the biotin tag using Western blot analysis is the use of high-resolution digital imaging techniques. The use of film is rarely optimal because the narrow linear range of chemiluminescence using film can result in image saturation. Digital camera imagers and fluorescence imagers (e.g., Typhoon imagers) have a wider dynamic range and are therefore recommended for Western blotting applications using biotin tags and/or in-gel fluorescence imaging.

Blotting protocol

Biotinylated protein samples are separated by 1D or 2D SDS–PAGE. To measure biotin content in experimental samples, biotinylated protein standards should be included on the gel. For 1D gels, a standard curve may be constructed by varying amounts of one biotinylated protein in a few lanes. For 2D gels, the biotinylated standard protein(s) can be run in a lane adjacent to the first-dimension gel strip. Duplicate gels can be run so that proteins from one gel can be stained with Sypro Ruby or comparable protein stains and proteins from the other gel can be transferred to nitrocellulose or PVDF and blotted for biotin detection. To quantify moles of biotin per mole of protein, nonbiotinylated protein can be mixed with biotinylated protein, so that the standard can be used to calibrate both stained gels and Western blots [10].

To blot proteins, transfer to nitrocellulose or PVDF membrane at 100 V for 2 h with cooling or at 25 V overnight at 4 °C. If using a Typhoon imager in later steps (see below), a low-fluorescence PVDF membrane such as Hybond-LFP should be used. Block nonspecific binding sites with 5% milk in TBS-T for 1 h. Wash membranes thoroughly to remove all milk. (*Note:* some milk blotting formulations contain biotin. Because this could interfere with streptavidin binding to biotinylated proteins, milk should not be included after the blocking step). Incubate blots with streptavidin–HRP (1:10,000 dilution in 10 ml of TBS-T) for 1 h. Wash membranes three times for 10 min each. Add chemiluminescent substrate evenly to the blot, ensuring coverage of the entire surface. For chemifluorescence imaging of biotin incorporation, use ECL Plus substrate and incubate on the membrane for 5 min under dim-lighting conditions or in the dark.

In-gel fluorescence imaging

If using fluorophore-tagged alkylating agents (e.g., BODIPY-IAM or -NEM), the image may be acquired directly from the gel. However, *it is very important to run the dye front completely off of the gel before imaging,* because the unreacted alkylating agent can interfere with the image and result in poor image quality in the lower half of the gel. Before imaging, wash the outside of the glass plates containing the gel with 70% ethanol, followed by distilled

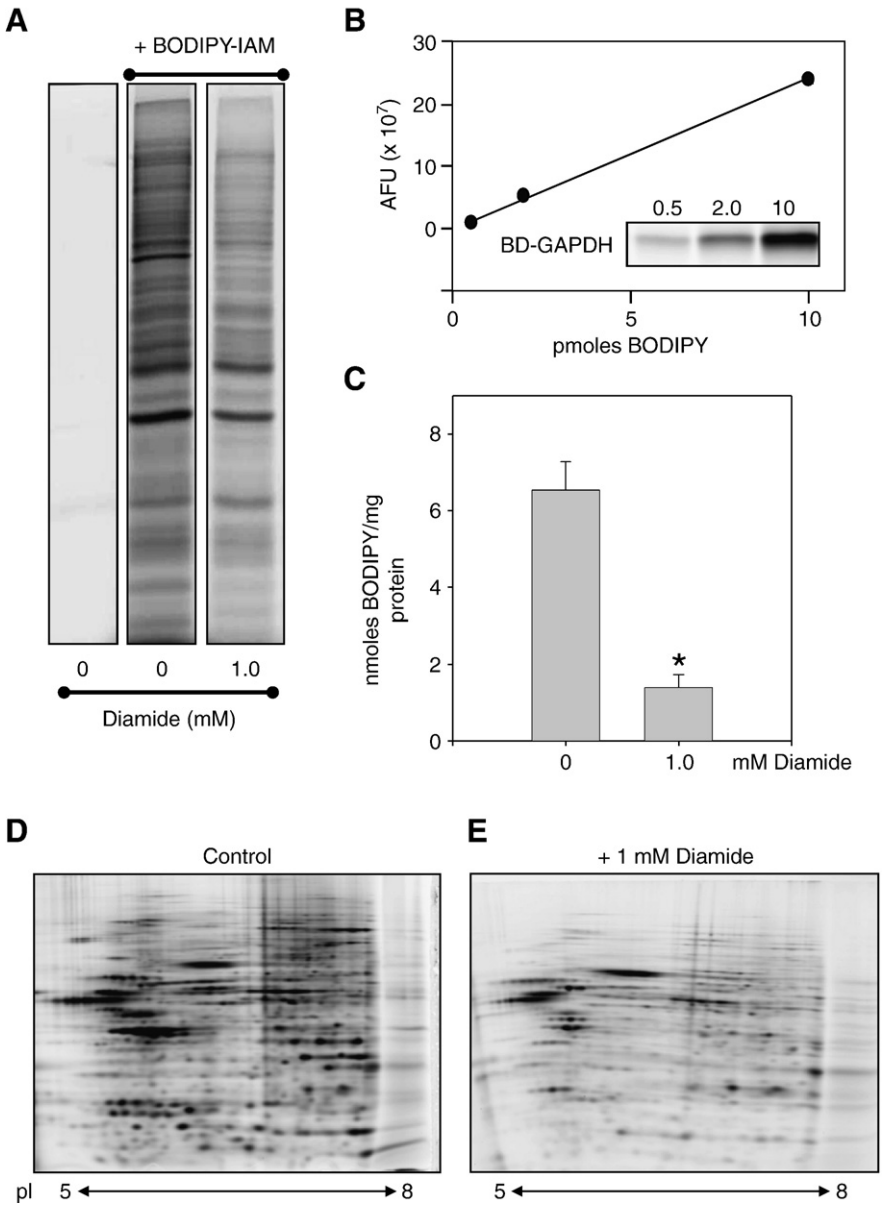

Fig. 6. Quantitation of thiol modifications using BD-GAPDH. (A) Mesangial cells were treated with vehicle (DMSO) or 1 mM diamide for 10 min. BODIPY-IAM (final concentration 50 μM) or vehicle was added to the cells and allowed to derivatize in situ for 30 min. The cells were then lysed and separated by 12% SDS–PAGE, and the BODIPY fluorescence was visualized in-gel using a Typhoon imager. (B) Three concentrations of BD-GAPDH were run in the same gel and used to construct a standard curve; $y = 2 \times 10^6 x$, $R^2 = 0.99$. (C) Quantification of the reduced thiol status in cells treated with 0 or 1 mM diamide. $n = 3$/group; $^*p < 0.005$. (D) 2D proteomic profile of BODIPY-modified proteins from control cells. (E) 2D proteomic profile of BODIPY-modified proteins in cells treated with 1 mM diamide.

water. It should be noted that standard glass plates often have intrinsic fluorescent properties that could affect image quality. In our experience, conventional glass plates do not show any background fluorescence on a Typhoon imager at the excitation and emission settings described below for the BODIPY-FL fluorophore. However, if fluorophores other than BODIPY-FL or multiple fluorophores with different spectral properties will be used, it is advisable to use low-fluorescence glass plates. Scan using the proper wavelength and emission filter settings; the excitation maximum for BODIPY-FL is at 505 nm and the emission maximum is at 513 nm. Refer to the respective instrument manual for guidelines on emission and excitation settings.

Western blot imaging

For Western blotting applications, acquire a series of images using a CCD camera imager (AlphaInnotech), a fluorescence imager (Typhoon; Amersham Biosciences), or similar instrument. For chemiluminescence imaging, the substrate should be left on the membrane during imaging. Imaging can be performed using a "movie" function, which integrates serial exposures. The result is a "movie strip" containing images of increasing intensity. Images should be saved as TIFF format files, which are used for subsequent analyses. Images containing saturated pixels should not be used for quantitation purposes.

For chemifluorescence imaging, the membranes should be dried before imaging or placed in distilled water and imaged while wet. In our experience using a Typhoon variable mode imager, less background is observed when the membrane is dried completely before imaging. The signal is stable for >48 h after development using ECL Plus detection reagents. After exposure to ECL Plus, transfer the membrane to a stacked layer of Kimwipes and gently blot the membrane dry. Afterward, place the membrane on a piece of filter paper and allow it to dry for at least 20 min before imaging. Failure to allow the blot to dry completely will result in uneven background and

image artifacts. Lay the blot face-down on the Typhoon platen and overlay with a piece of nonfluorescent plastic such as 3M Dual Purpose transparency film. Scan the image in the fluorescence acquisition mode using the 520 BP 40 emission filter and the blue (488 nm) laser. (*Note:* if using a Typhoon Trio imager, use the ECL + excitation setting.) Set the PMT voltage to 400–450 V initially. Do not press the sample; the plastic transparency film will adequately press the membrane against the platen. Set the pixel size to 500–1000 μm initially. Adjust the PMT voltage to obtain an image that is intense but not saturated. Take the final image using a pixel size of 100 μm.

Acquire Sypro Ruby images using UV light if using a CCD camera imaging system or with Sypro Ruby filter settings and the appropriate laser conditions if using a Typhoon imager.

Analysis

The amount of biotin is quantitated by determining the density (in arbitrary units) of the selected area with AlphaEaseFC software if using a CCD camera or with ImageQuant TL software if using the Typhoon imager. Several alternative densitometry programs exist and can be used in a manner similar to those described here. For 1D gels, the densities of each lane containing experimental samples and biotinylated standard proteins are determined. Note that background subtraction settings are particularly important here, and, in our experience, the "minimum profile" setting is optimum for assessment of overall biotinylation or BODIPY signal from 1D membranes or gels.

Advantages and caveats

This protocol describes the use of alkylating agents for the detection of reactive protein thiols. Depending on the intent of the experiment or project, other types of probes may also be used to interrogate the thiol proteome. For instance, disulfide-forming probes or probes similar to those used in protein spin labeling studies such as biotinylated glutathione, methyl methanethiosulfonate, or *N*-(6-(biotinamido)hexyl)-3′-(2′-pyridyldithio)propionamide may be used alone or in concert to detect specific protein modifications [1,2,5–8,24]. The advantage to using these probes is that particular reductants or derivatization reagents (e.g., ascorbate [24] or dimedone [25]) allow for detection and discrimination of specific reversible posttranslational protein modifications such as S-nitrosated or sulfenic acid-modified proteins. The protocols described here, however, focus mainly on identifying the overall proteome that is modified. It should be noted that these protocols could easily be adapted and used jointly with other fluorophore-labeled probes and protocols to give further insight into and to quantify potentially important protein thiol modifications. For example, it is possible to extend this protocol not only to detect the overall thiol proteome that is modified but also to discern the proportion of those proteins that are S-nitrosated, -glutathiolated, or -oxidized.

The choice of alkylating reagent depends on the experimental intent and the imaging facilities available. IAM will alkylate many reduced protein thiols at high pH; alternatively, NEM can be used to alkylate for this purpose at neutral pH or below. To label thiols that are most highly reactive (i.e., those that have a low pK_a), IAM should be used at neutral pH or below. These thiols, which make up the reactive thiol proteome, are of interest in most protein modification studies because of their potential involvement in redox signaling or their role in pathology owing to modification during excessive oxidant stress. The caveat to using iodo derivatives is incomplete alkylation of the reactive thiol proteome. In studies by Rogers et al., NEM was shown to alkylate thiols more fully than eightfold higher concentrations of IAM or IAA [12]. Hence, the amount of thiols modified by IAM or IAA per milligram of protein will probably be an underestimation of the actual reactive thiol proteome. Because of this, measurement of thiols using this protocol is not absolute. Nevertheless, if all samples are treated equally, the measurements are quantitative and exceed more semi-quantitative measurements based only on assessing arbitrary or relative fluorescence units. The external standards used in this protocol are the primary advancement over previous methods. In our experience, the fluorescence standards have a much wider linear dynamic range and can be detected in femtomole quantities.

Acknowledgments

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