

Original Contribution

# A sensitive method for the quantitative measurement of protein thiol modification in response to oxidative stress<sup>☆</sup>

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## Abstract

The combination of proteomics with highly specific and sensitive affinity techniques is important for the identification of posttranslational modifications by reactive oxygen and nitrogen species (ROS/RNS). One of the most pressing problems with this approach is to determine accurately the extent of modification of specific amino acids, such as cysteine residues, in a complex protein sample. A number of techniques relevant to free radical biology use biotin tagging as a method to follow protein modification with high sensitivity and specificity. To realize the potential of this approach to provide quantitative data, we have prepared a series of biotinylated proteins through the modification of lysine residues. These proteins were then used as quantitative standards in electrophoretic separation of protein samples labeled with biotin-conjugated iodoacetamide. The utility of the approach was assessed by measuring modification of thiols in response to exposure to thiol oxidants, as well as the amount of protein adduct formation with a biotin-tagged electrophilic lipid. Furthermore, using a combination of native and biotin-tagged cytochrome *c*, this method was used to quantitate the amount of thiol relative to the amount of protein in a given spot on a two-dimensional gel. Thus, we have developed a versatile, cost-effective standard that can be used in proteomic methods to quantitate biotin tags in response to oxidative stress.

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**Keywords:** Thiol proteomics; Diamide; 4-Hydroxynonenal; Oxidative stress; Biotin; Free radicals

## Introduction

With the widespread availability of high-resolution formats for the separation of complex mixtures of proteins, it is now possible to monitor changes in the proteome of a range of biological samples in response to a variety of experimental conditions [1–5]. A number of approaches are available for the identification of protein spots from a 2D-format including mass spectrometry directly from the stained gel or Western blotting if an antibody to a specific protein is available. Western blotting techniques can be viewed as complementary to mass spectrometry approaches since, in addition to providing evidence to support the assignment of a protein identity, they can also provide quantitative information. This is particularly important in the field of redox cell signaling since it is now recognized that the oxidative or nitrosative modification of proteins can lead to control of cell function [6–9].

The posttranslational modification of proteins by ROS/RNS is now emerging as an important mechanism through which

**Abbreviations:** 15d-PGJ<sub>2</sub>, 15-deoxy-Δ<sup>12,14</sup>-prostaglandin J<sub>2</sub>; BAEC, bovine aortic endothelial cells; HUVEC, human umbilical vein endothelial cells; BIAM, *N*-(biotinoyl)-*N*-(iodoacetyl)ethylenediamine; bt-cyt *c*, biotinylated cytochrome *c*; bt-SBTI, biotinylated soybean trypsin inhibitor; bt-Mb, biotinylated myoglobin; 5,5'-DTNB, dithio-bis-(2-nitrobenzoic acid); DTT, dithiothreitol; GSH, glutathione; HABA, 2-(4'-hydroxyazobenzene) benzoic acid; 4-HNE, 4-hydroxynonenal; IEF, isoelectric focusing; MALDI-TOF, matrix-assisted laser desorption/ionization time of flight; PBS, phosphate-buffered saline; RNS, reactive nitrogen species; ROS, reactive oxygen species; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; sulfo-NHS-LC-biotin, sulfosuccinimidyl-6-(biotin-amido) hexanoate.

<sup>☆</sup> This paper is dedicated to the memory of Dr. Amanda Isom who tragically lost her life to an automobile accident in April 2005.

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protein function can be monitored during the complex pathologies associated with a variety of disease states including inflammation [10,11]. Of particular interest are modifications of thiol residues, since it has long been recognized that cysteine can play an important role in controlling the activity of a wide variety of enzymes and cell-signaling proteins [7,12–14]. The techniques available to detect oxidative modifications for low abundance proteins include affinity methods using antibodies or other molecular tags [15–22]. Of these one of the most versatile is the use of biotin which can be attached covalently to a number of reactive molecules including oxidized lipids and thiol-reactive reagents [16,21,23–27]. After separation by proteomics methods the biotin tag can be detected at a level of sensitivity in the picomole range using Western blotting with HRP-conjugated streptavidin. An additional advantage to using biotin tags is that, in principle, the extent of protein modification can be quantified. This is important since the degree of posttranslational modification of a protein is generally an important predictor of the biological impact of regulation through this mechanism. Biotin-based tagging methods have been applied with some success to the detection of reactive protein thiols in cell-signaling proteins and enzymes [16,21,23,28].

While existing methods using high-resolution proteomics formats can readily determine if protein oxidation has occurred or not it is difficult to assess the extent of modification on a molar basis. On the other hand, mass spectrometry methods in which this can be achieved are not usually capable of assessing all peptides within a protein. In addition advanced mass spectrometry methods are not broadly available to most investigators interested in screening biological samples from a complex experimental matrix. It is likely that 2D polyacrylamide gel-based proteomics formats will remain a complementary approach to mass spectrometry for many routine applications. In this setting quantitative assessment of protein modification is important, since it is frequently the case that a protein may have several potentially reactive amino acid residues but modification of only one site leads to significant change in function. To provide a practical approach to solving the quantitation problems associated with the application of Western Blotting to proteomics, we have developed a method for the synthesis and of an internal standard. To achieve this, we have biotinylated a number of proteins and characterized the reaction to achieve a precise number of biotin molecules per molecule of protein. Using model systems in both cells and intact mitochondria the characterization, preparation, and application of the bt–protein standards are described and their use in assessing the modification of thiol residues under conditions of oxidative stress is demonstrated.

## Experimental procedures

### Materials

All chemicals were purchased from Sigma-Aldrich-Fluka (St. Louis, MO) except as noted.

### Preparation of biotinylated proteins

Bt-cyt *c* was prepared by reacting horse heart cytochrome *c* (10 mg/ml in PBS) with sulfo-NHS-LC-biotin (Pierce, Rockford, IL). For the initial series of experiments, the molar ratio varied from 0 to 20 mol biotin per mole cytochrome *c*. The reaction was allowed to proceed for 4 h on ice, and unreacted biotin was removed using a P-10 gel-filtration column. The red cytochrome *c* band was collected and the biotin incorporation determined in this fraction using a colorimetric HABA dye displacement assay (Pierce). The protein concentration of the cytochrome *c* fraction was determined using the absorbance of the dithionite-reduced protein at 550 nm using an extinction coefficient of  $27.6 \text{ mM}^{-1} \text{ cm}^{-1}$ . To determine the biotin content the change in absorbance of 0.9 ml of avidin-HABA solution (0.5 mg/ml avidin and 0.3 mM HABA in PBS) was measured at 500 nm upon addition of 0.1 ml of a bt-cyt *c* sample. In the linear range of the assay, an extinction coefficient of  $34 \text{ mM}^{-1} \text{ cm}^{-1}$  was used to quantitate biotin. Mass spectrometry and experiments using bt-cyt *c* as a quantitative standard were performed with bt-cyt *c* containing approximately 2–3 mol biotin/mol cytochrome *c*. In addition to cytochrome *c*, proteins of various molecular weights including horse heart myoglobin (Mb), and soybean trypsin inhibitor (SBTI) were reacted with biotin as described above for cytochrome *c* with the exception that the excess biotin and other reagents were removed by dialysis. For these samples the protein content was determined by the Lowry protein assay. The biotin content was again determined from the HABA assay as described above for bt-cyt *c*.

### Mass spectrometry

Undigested native and bt-cyt *c* were analyzed by MALDI-TOF MS to detect changes in total protein mass [29]. Briefly, samples were mixed 1/10 (v/v) with a saturated solution of sinapinic acid in acetonitrile/0.1% aqueous trifluoroacetic acid (1/1, v/v) and 1  $\mu\text{l}$  was spotted onto the MALDI target plate. Protein molecular ions were analyzed in linear, positive-ion mode using a Voyager Elite mass spectrometer (Applied Biosystems, Inc., Foster City, CA). Each spot was analyzed a minimum of three times, each spectrum consisting of 200 laser shots. The resulting spectra were analyzed by DataExplorer (Applied Biosystems, Inc.) and the instrument calibrated using an external apomyoglobin standard. The resulting spectra were Gaussian-smoothed and baseline-corrected.

Tryptic and chymotryptic peptide maps were used to identify the peptides to which an addition of biotin had occurred by comparison of *m/z* shifts with those from unmodified cytochrome *c*. For peptide mass fingerprinting, peptide mass maps were compared between native and bt-cyt *c* samples in order to identify *m/z* shifts of 339 units.

### Mitochondrial preparation

Five- to 7-week-old male wild-type (C57BL/6) mice were obtained from Jackson Laboratory (Bar Harbor, ME), housed in a conventional facility, and maintained under a 12 h light/

dark cycle. Liver mitochondria were prepared and characterized as previously described [30,31]. All animals were handled in accordance with recommendations in *The Guide for the Care and Use of Laboratory Animals*.

#### *Exposure of samples to oxidants, proteomics, and Western blotting*

Isolated mouse liver mitochondria were separated by both conventional SDS-PAGE and 2D-IEF. In one series of experiments isolated mitochondria were treated with 40  $\mu$ M Angelis salt (AS) for a period of 15 min under conditions which we have previously shown results in extensive modification or protein thiols [32]. In cell-based experiments confluent bovine aortic endothelial cells (BAEC) were prepared as described in [33]. Cells were then exposed to 4-HNE (40  $\mu$ M) or diamide (1 mM) for 1 h. After incubation cells were lysed in the presence of biotin-conjugated iodoacetamide (*N*-(biotinoyl)-*N*-(iodoacetyl)ethylenediamine) (BIAM, 100  $\mu$ M) in order to label free sulfhydryl groups on cellular proteins [16]. In addition DTNB (1 mM) was also used to measure total cellular free thiol groups at 412 nm and total GSH was measured using the Tietze recycling assay with modifications as described in [34]. For experiments tracking the reactivity of an electrophilic lipid, biotinylated 15d-PGJ<sub>2</sub> (15-deoxy- $\Delta^{12,14}$ -prostaglandin J<sub>2</sub>) was prepared as described [21]. HUVEC were treated with 20  $\mu$ M bt-15d-PGJ<sub>2</sub> for 4 h, and cell lysate was analyzed by Western blot analysis as described below.

Isolated mitochondria treated with or without Angelis's salt (Alexis, San Diego, CA) were lysed in a buffer containing 50 mM 2-(*N*-morpholino)ethanesulfonic acid-NaOH (pH 8.5), 1% Triton X-100 with Complete Mini protease inhibitor cocktail (Roche), followed by labeling with BIAM (100  $\mu$ M) for 15 min in the dark and terminated by the addition of  $\beta$ -mercaptoethanol (20 mM). Samples were then separated by conventional SDS-PAGE (10–15% gradient acrylamide gels) before transfer to nitrocellulose paper and Western blotting. After blocking, the sample was probed with streptavidin-HRP (Amersham Biosciences, Piscataway, NJ) and developed using a chemiluminescence imager (AlphaInnotech, San Leandro, CA). Parallel gels were stained with Sypro Ruby (Molecular Probes, Eugene, OR) to visualize protein.

For two-dimensional electrophoresis, the isolated mitochondria were diluted with rehydration buffer containing 40 mM Tris-HCl, pH 8.8, 7 M urea, 2 M thiourea, 30 mM dithiothreitol, 2% cholamidopropyl-diethylammoniumpropane sulfonate, 0.5% lauryl maltoside, and 2% ampholytes, pH 3–10. The rehydrated IPG strips were focused at 4000 V for 20,000 V-h on the Invitrogen (Carlsbad, CA) ZOOM runner IEF system. After IEF, the strips were equilibrated for 20 min in 50 mM Tris-HCl, pH 8.8, 6 M urea, 20% glycerol, 2% SDS, trace bromophenol blue, and 50 mM DTT. Second-dimension SDS-PAGE was performed on 10–18% gradient polyacrylamide gels. A sample of known concentration of bt-cyt *c* (3.5 pmol biotin, 0.01  $\mu$ g protein) was supplemented with 0.31  $\mu$ g native cytochrome *c* and introduced in a lane adjacent to the IEF sample on the second-dimension SDS-PAGE gel. Samples

were then either stained for protein using Sypro Ruby or transferred to nitrocellulose paper for Western blotting and detection of biotin as described above.

#### *Quantitation of biotin and protein in 1D and 2D gels*

Images of Western blots and Sypro Ruby gels were taken using a CCD camera imager (AlphaInnotech) and saved in TIFF format files, which were used for subsequent analyses. For all experiments, images which did not have saturated pixels were chosen. For 1D gels, density was determined for with the AlphaEaseFC software version 4.0.0 supplied by the manufacturer of the camera using the 1D-Multi analysis tool. The amount of density corresponding to pmoles biotin was determined for each protein relative to the intensity determined from bt-cyt *c* since the extent of biotinylation was determined with a high degree of accuracy both by mass spectrometry and by the HABA assay. The integration of each band for the individual proteins was then used to construct a standard curve. For 2D gels, density was determined using the Spot Denso analysis tool for the bt-cyt *c* or unlabeled cytochrome *c*, and the amount of density corresponding to the picomoles biotin and picomoles protein calculated. This value was then used to determine the amount of protein and biotin in each spot of interest.

## **Results and discussion**

### *Preparation and characterization of biotinylated proteins*

Cytochrome *c* and myoglobin and soybean trypsin inhibitor (10 mg/ml) were biotinylated via lysine residues under nondenaturing conditions by reaction with 0- to 20-fold molar excess of sulfo-NHS-LC-biotin at 4°C for 4 h. Biotinylated proteins can be stored at 4°C in the presence of protease inhibitors for several weeks. In the case of bt-cyt *c*, freezing and dilution into nondenaturing buffers led to irreversible aggregation of the protein over several hours. For quantitation of the extent of biotin incorporation into cytochrome *c*, the dye HABA was used which forms a colored complex with avidin and is readily displaced by biotin, leading to a proportional decrease in absorbance at 500 nm. Using this assay, we determined that the amount of biotin incorporated into bt-cyt *c* can be controlled by modification of the reaction conditions (Fig. 1A). In this series of experiments, the same samples were subjected to SDS-PAGE followed by Western blot analysis, and the amount of biotin incorporation by Western blot (Fig. 1A, inset) was consistent with the results obtained using the HABA assay. The fainter band migrating at 24 kDa is due to small amounts of cytochrome *c* dimer present in these preparations. The signal obtained from a streptavidin-HRP blot increases linearly with increasing amounts of bt-cyt *c* loaded onto the gel (Fig. 1B). It is also evident from these data that as little as 1 pmol biotin can be readily detected on the gel. However, the dynamic range for linearity of response using these techniques is highly dependent on the equipment available to the investigator. For example, in this study images

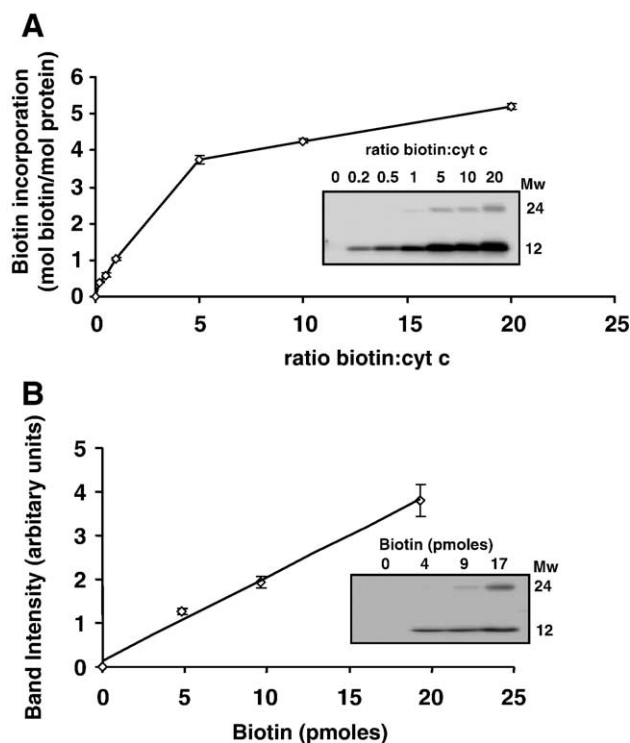


Fig. 1. Characterization of biotinylated cytochrome *c*. (A) Cytochrome *c* (10 mg) was reacted with different concentrations of sulfo-NHS-LC-biotin, and incorporation of biotin determined using the HABA dye displacement assay. Biotin incorporation was confirmed by Western blot using streptavidin-HRP (inset). (B) Quantification of band intensity from a standard curve of bt-cyt *c* determined at a moderate level of biotin incorporation (4.3 mol biotin/mol protein). A linear correlation between band intensity and biotin is shown with  $R^2 = 0.9915$  (blot shown in inset). Data are represented as the mean of independent measurements,  $n = 3 \pm \text{SE}$ .

were obtained using a CCD camera imager (Alpha-Innotech) using software which allows multiple consecutive images to be obtained from several samples under the same conditions. As with other approaches, using image analysis, data can be both quantitative and sensitive if due care is taken to validate the chosen experimental conditions.

To determine the sites and extent of cross-linking of biotin to cytochrome *c*, mass spectrometry was used. When compared with native cytochrome *c*, the MALDI-TOF spectrum of bt-cyt *c* revealed a distribution of products increasing by increments of  $m/z = 339$  (Fig. 2). The only exception was found for the peak at 12733, which differed from the next biotinylated form by  $m/z = 301$ , due to formation of a potassium adduct. In reasonable agreement with the HABA assay, the majority of the modified proteins were found with 2–5 biotin molecules attached, while unmodified cytochrome *c* was not detected.

Since the biotinylating reagent reacts with lysine residues, it was necessary to use chymotrypsin, which cleaves at hydrophobic residues, to prepare peptides for analysis, in addition to trypsin, to maximize coverage of the protein. The peptides were analyzed by MS/MS in order to confirm the exact position of the biotin tag. A total of 9 modified lysines were identified using this method: K5, K7, K8, K26, K39, K79, K86, K87, K88.

In the next series of experiments a series of biotinylated proteins were synthesized of differing molecular weight and subjected to SDS-PAGE and Western blotting for the detection of the biotin tag (Fig. 3). From the characterization of these proteins it is clear that a range of internal standards of different molecular weights can be generated using this method (Table 1). As with bt-cyt *c*, the intensity of the signal from the Western blot is linear with the concentration of biotin applied to the gel (Figs. 3A and B). This approach allows the mixing of the biotinylated proteins of different molecular weight to construct a standard curve within one lane of an SDS-PAGE gel (Figs. 3C and D). However, in order to illustrate the concept of using biotinylated internal standards, the following series of experiments were performed using bt-cyt *c* alone.

#### Measurement of oxidative stress in isolated mitochondria

As a model system for demonstrating the applicability of this method, we selected mitochondria since they are a known source of reactive oxygen species in the cell and the proteins can be modified under a broad range of pathological conditions [1]. Accordingly, bt-cyt *c* was used as an internal standard for the quantification of thiol modification in isolated murine liver mitochondria. As a model oxidative stress we used the nitroxyl donor Angeli's salt which we have shown modifies mitochondrial thiols leading to change in the function of complex II [32]. Mitochondria were incubated with or without 40  $\mu\text{M}$  AS. Protein thiols were then tagged with a thiol-specific alkylating agent, BIAM, which contains a biotin group as described in the methods section. After reaction, samples were prepared for SDS-PAGE with bt-cyt *c* (3.5 pmol biotin) included in a separate lane on the gel before detection by streptavidin conjugated to HRP. The resulting bands detected by HRP-streptavidin are shown in Fig. 4A, and it is clear that even under the low resolution of a 1D-SDS-PAGE there is a decrease in signal consistent with the modification of reduced thiols by AS. The incorporation of the bt-cyt *c* standard on the same blot enabled the absolute quantitation of these data (Fig. 4B). Integration of the total band intensity in the control sample results in a value of approximately 1.6 pmol thiol/ $\mu\text{g}$  protein. Treatment with AS decreased this value by 40% to 1.0 pmol/ $\mu\text{g}$  which is in agreement with the known ability of AS to react with thiols [32].

#### Detection of thiol modification on exposure of BAEC to oxidants

Next, we determined the applicability of this method for measuring the oxidation of thiols in more complex biological systems, in this case cultured bovine aortic endothelial cells exposed to oxidants. Confluent cells were exposed to either diamide (1 mM) or 4-hydroxynonenal (4-HNE, 40  $\mu\text{M}$ ) for a period of 1 h before a cell lysate was prepared. Diamide is an oxidant that promotes S-thiolation reactions, and 4-HNE is a reactive aldehyde associated with increased oxidative stress in vivo [17,35] (Fig. 5A). Thiols were labeled with BIAM as above, and proteins separated by 1D-SDS-PAGE. Quantitation

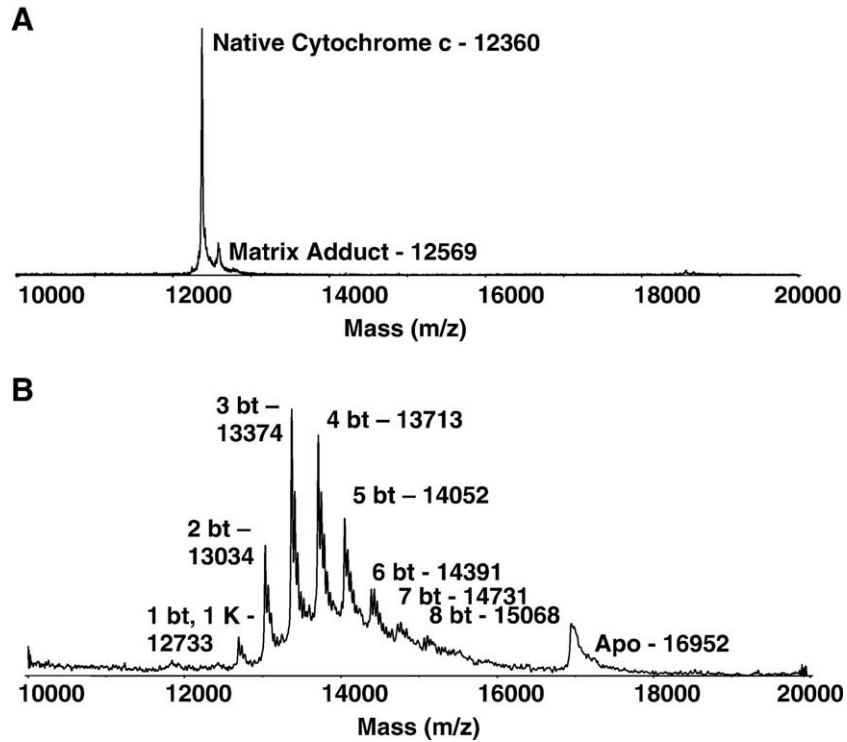


Fig. 2. Mass spectra showing shifts in cytochrome *c* molecular weight due to biotinylation. (A) MALDI-TOF mass spectrum of native cytochrome *c* with internal calibration on the intact mass of cytochrome *c*. (B) MALDI-TOF mass spectrum of bt-cyt *c* with internal calibration using intact apomyoglobin.

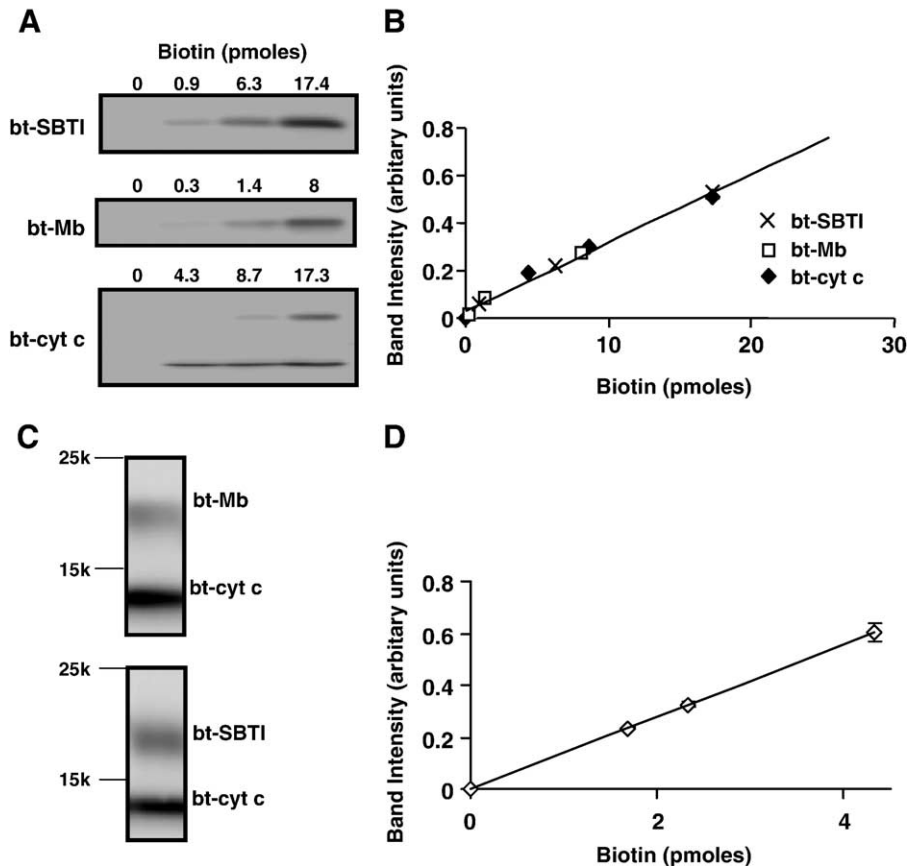


Fig. 3. Biotinylation of proteins of various molecular weights. (A) Biotin incorporation of proteins with different molecular weights was determined by Western blot analysis using streptavidin-HRP. (B) A linear relationship exists between band intensity and biotin level with  $R^2 = 0.9939$ . (C) All biotinylated proteins were run in a single lane to generate a standard curve (D).

Table 1  
Biotinylation of proteins of varying molecular weight

	Molecular weight (kDa)	Biotin/protein		Lysine residues /mol
		mol/mol	nmol / $\mu$ g	
bt-cyt c	12.4	4.8 $\pm$ 0.2	0.4 $\pm$ 0.02	19
bt-Mb	17	3.3 $\pm$ 0.1	0.2 $\pm$ 0.003	19
bt-SBTI	21.5	5.0 $\pm$ 0.2	0.2 $\pm$ 0.01	8

Values are mean  $\pm$  SE ( $n = 3$ ). The protein concentration of bt-cyt c was determined spectrophotometrically at 550 nm. All other biotinylated protein concentrations were determined by the Lowry assay. Biotin incorporation into the proteins was measured using the HABA assay.

of each of these lanes by densitometry, and with reference to the bt-cyt c, allowed the extent of biotin incorporation before and after exposure to the oxidants to be calculated (Fig. 5B). After 1 h approximately 35% of the protein thiols were modified in the case of 4-HNE and 45% in the case of diamide. We also used independent methods to measure both protein thiols and glutathione in the same samples. Free thiols can be measured directly at 412 nm after reaction with

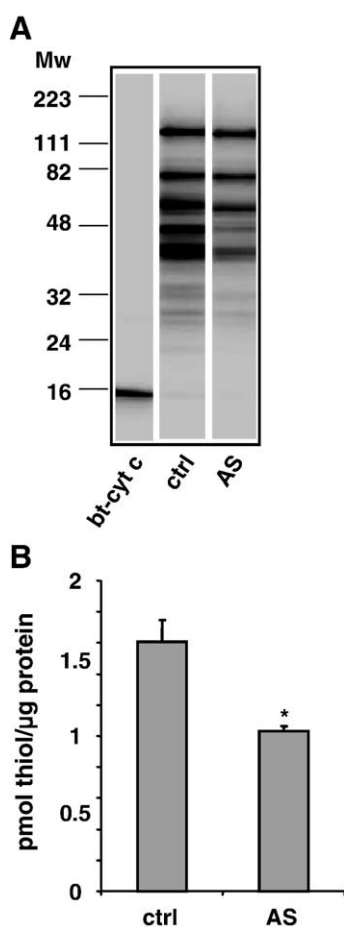


Fig. 4. Quantitation of mitochondrial protein thiols by Western blot analysis. (A) Isolated mouse liver mitochondria were treated with vehicle (control) or 40  $\mu$ M Angeli's salt, followed by labeling of free thiols using BIAM (100  $\mu$ M). Mitochondrial proteins (10  $\mu$ g/lane) were separated by SDS-PAGE, and biotin was detected by Western blot using streptavidin-conjugated HRP. (B) Biotin incorporation was determined using bt-cyt c as a standard. Results are expressed as means  $\pm$  SE,  $n = 3$ . \* $P < 0.01$  relative to control.

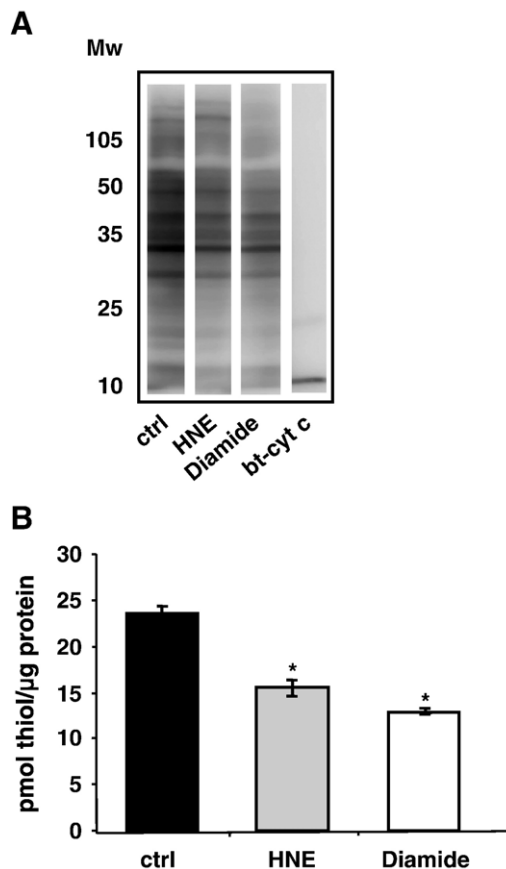


Fig. 5. Quantitation of the effect of oxidants on thiol modification. (A) BAEC were treated with HNE (40  $\mu$ M) or Diamide (1 mM) for 1 h. Cells were lysed and labeled with BIAM (100  $\mu$ M). Samples (10  $\mu$ g protein) were run on an SDS-polyacrylamide gel followed by Western blot analysis. (B) BIAM incorporation in each sample from panel A was quantified using bt-cyt c as a standard (6.7 pmol biotin). Results are expressed as mean  $\pm$  SE,  $n = 3$ . \* $P < 0.002$  relative to control.

DTNB (dithionitrobenzoate) and GSH can be measured specifically through the addition of glutathione reductase [34]. From these measurements protein thiol levels can be calculated and were found to be approximately 100 pmol/mg protein which, as expected is 4-fold greater than that determined from the BIAM labeling. The lower value is consistent with selectivity and lower efficiency for thiol labeling by the BIAM reagent through an alkylation reaction when compared to the rapid and facile disulfide exchange reaction with DTNB.

#### Detection of low abundance lipid–protein adducts

In the previous approach the loss of the biotin signal from the tagged protein thiols was demonstrated upon oxidative stress. A further application of the biotin tagging methodology is to modify a reactive molecule of interest and then detect the formation of products. This method has been used for the detection of the adducts formed between electrophilic lipids such as 15-deoxyprostaglandin  $J_2$  and proteins in several different cell types [21,28]. Protein–lipid adducts are formed via Michael addition between nucleophilic protein residues

such as cysteine and electrophilic carbon centers in 15d-PGJ<sub>2</sub> (Fig. 6A, denoted by asterisks). In this experiment, human umbilical vein endothelial cells (HUVEC) were incubated with biotin tagged 15d-PGJ<sub>2</sub> for 4 h before preparation of a cell lysate and detection of the protein adducts as described above. A number of biotin-tagged proteins are evident after exposure to 15d-PGJ<sub>2</sub> that are not present in the control sample (Fig. 6B). The low intensity band present in the control is due to the detection of an endogenous biotin-containing carboxylase. For this low abundance level of biotinylation, the concentration of bt-cyt c used as the normalizing standard on the gel was approximately 10 times lower than that used for the total thiol detection experiment (Figs. 3 and 4). In this case, approximately 1.2 pmol of electrophilic lipid/ $\mu$ g protein was found adducted to proteins in the cell after correction for endogenous biotin. These

experiments show the wide concentration range over which bt-cyt c can be used as an internal standard.

#### Application of bt-cyt c to high-resolution proteomics

An advantage of the inclusion of the internal bt-cyt c standard is that samples with varying levels of signal can be compared on a quantitative basis. This can be demonstrated using high-resolution proteomics techniques, and in this case we have applied the bt-cyt c standard to quantitate reduced thiols in specific proteins from isolated liver mitochondria using BIAM. For these experiments, we prepared a mixture of native cytochrome c and bt-cyt c which could be used as a dual internal standard to quantitate individual proteins and simultaneously measure the thiol content. Total thiols in a mitochondria were labeled with BIAM, and separated by IEF followed by SDS-

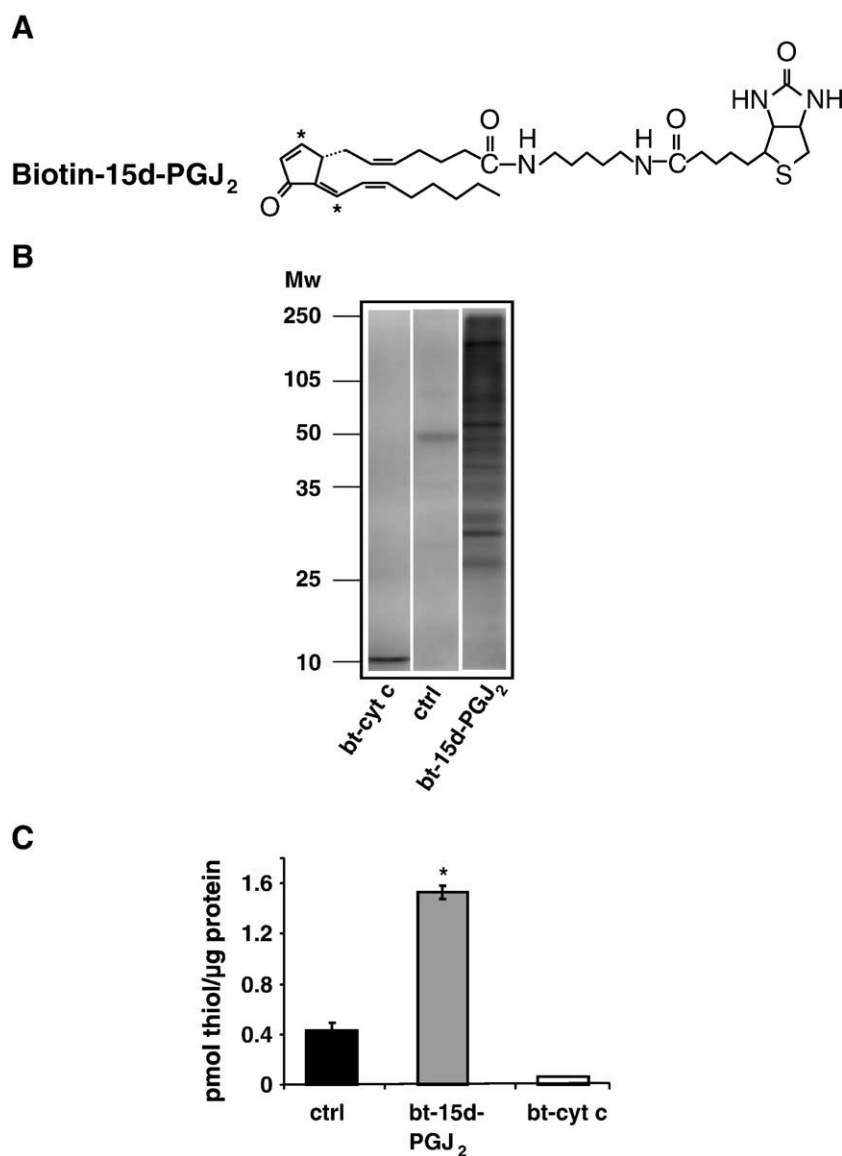


Fig. 6. Quantitation of the effect of electrophilic lipids on thiol modification. (A) Structure of bt-15d-PGJ<sub>2</sub>. (B) HUVEC were treated with bt-15d-PGJ<sub>2</sub> (20  $\mu$ M) for 4 h and cell lysates analyzed by Western blot using streptavidin-HRP. Electrophilic lipid–protein adducts were observed as an increase in the biotin signal. (C) The amount of biotin labeling was quantitated using bt-cyt c (0.67 pmol biotin). Results are expressed as mean  $\pm$  SE,  $n = 3$  for control and lipid-labeled samples. \* $P < 0.0001$  relative to control.

PAGE. In the second-dimension SDS-PAGE, the native/bt-cyt *c* was mixed with the marker and run in a separate lane beside the IEF strip. The bt standard was then run in the second-dimension under conditions identical to those of the sample. The incorporation of the bt standard into the sample at the time of isoelectric focusing is not recommended since the modification of the lysine residues results in a trail of spots with different isoelectric points across the second-dimension gel. This makes quantitation difficult and interferes with the pattern of spots from the sample itself. Duplicate gels were either stained with Sypro Ruby for protein (Fig. 7A) or transferred to nitrocellulose, and the BIAM-reactive proteins detected with streptavidin-HRP as described above (Fig. 7B). The proteins labeled 1, 2, and 3 show spots present in both the protein stained gel and the BIAM Western blot. In order to confirm the overlay of the blot image with the gel, the protein corresponding to spot 1 was identified as ATP synthase  $\beta$ -subunit by peptide mass fingerprinting and by Western blot analysis (data not shown). It is clear from visual inspection of the spot patterns for the BIAM-stained gel and protein that the patterns differ substantially. This is expected since the intensity of a signal from a BIAM-reactive spot reflects the abundance of the protein, number of thiols, and reactivity to BIAM, and does not necessarily correspond to intensity determined from protein staining. Proteins selected for further analysis were visualized on both the gel and the blot and shown in the magnified image below (Figs. 7A and B).

Next, the Sypro protein stain for the bt-cyt *c* was calculated and used as a standard to normalize each protein spot on the gel. This approach is essentially the same as using a protein standard in a routine protein assay and is constrained by the assumption that the amount of Sypro dye bound per mole protein is constant. We chose to investigate well-characterized proteins with known thiol content for our study: aldehyde dehydrogenase and glutamate dehydrogenase. The identity of these spots was confirmed using peptide mass fingerprinting (data not shown). The signal from the bt-cyt *c* was equivalent to 3.5 pmol biotin. The amount of thiol in the spot identified as aldehyde dehydrogenase was  $5.7 \pm 0.83$  (SD) mol biotin-labeled thiol/mol protein for  $n = 2$  replicate gels. Similarly, the amount of thiol in another protein spot, glutamate dehydrogenase, was  $2.7 \pm 0.02$  (SD) mol biotin-labeled thiol/mol protein, for  $n = 2$  replicate gels. From the amino acid sequences of these proteins the total moles thiol/moles protein was determined to be 10 and 6 for aldehyde dehydrogenase and glutamate dehydrogenase, respectively. Thus, we were able to label approximately 50% of the total thiols in these proteins. The fact that not all of the thiols were labeled is not surprising, since the labeling is dependent on the state of endogenous thiol oxidation, as well as the pH of the labeling reaction, the  $pK_a$  of the thiol, and accessibility of each individual cysteine residue to the BIAM reagent. However, this level of labeling has been sufficient for us to measure thiols in a physiologically relevant range under normal conditions and in response to oxidative stress.

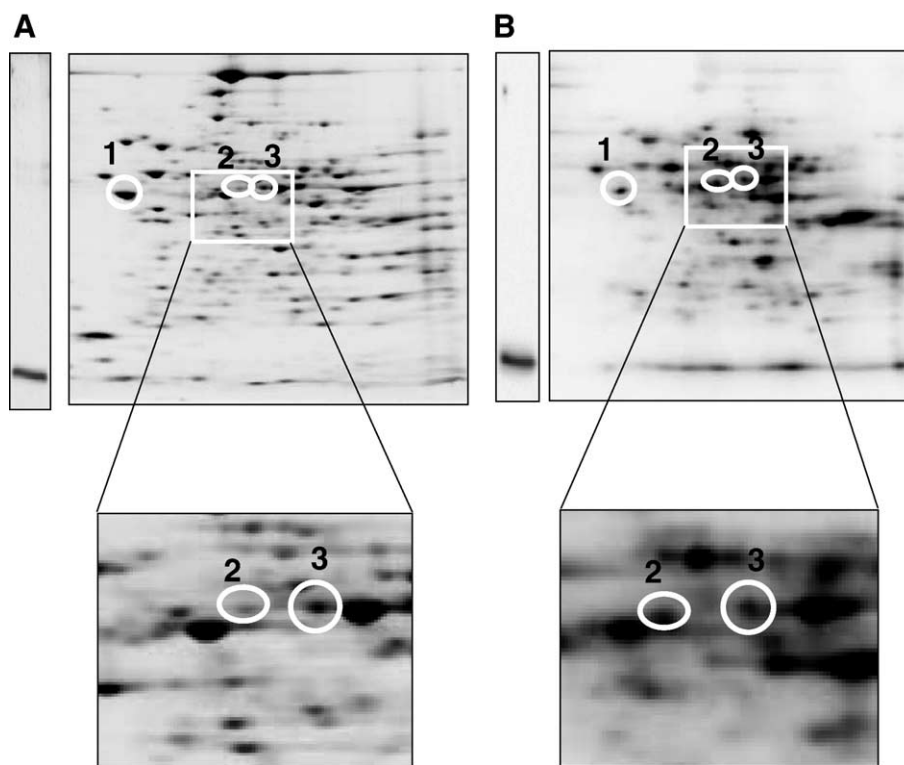


Fig. 7. Quantitation of protein thiols in individual proteins after two-dimensional electrophoresis. Protein thiols were labeled with BIAM prior to separation by two-dimensional electrophoresis. A mixture of native cytochrome *c* (0.3  $\mu$ g) and bt-cyt *c* (0.01  $\mu$ g, 4.2 pmol biotin) was added to a lane alongside the sample. (A) Total protein was visualized on one gel using Sypro Ruby. (B) Proteins from an identical gel were transferred to nitrocellulose, followed by detection of the biotin label by Western blot using streptavidin-HRP. The lower insets show boxed regions of the gel in more detail. Circles indicate the spots corresponding to (1) ATP synthase  $\beta$ -subunit, (2) aldehyde dehydrogenase, and (3) glutamate dehydrogenase.

## Summary

In this study we have shown how readily available techniques can be used to prepare an internal standard for biotin tagging from the controlled reaction of commercially available proteins with a biotin label. The resulting adducts are stable and are sufficiently sensitive to detect posttranslational modification of proteins at the picomole level required for proteomics applications in free radical biology.

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