Protein Sulfenation as a Redox Sensor

PROTEOMICS STUDIES USING A NOVEL BIOTINYLATED DIMEDONE ANALOGUE

Rebecca L. Charles‡, Ewald Schröder‡, Georgina May§, Paul Free§, Piers R. J. Gaffney§, Robin Wait¶, Shajna Begum¶, Richard J. Heads‡, and Philip Eaton‡

Protein sulfenic acids are reactive intermediates in the catalytic cycles of many enzymes as well as the in formation of other redox states. Sulfenic acid formation is a reversible post-translational modification with potential for protein regulation. Dimedone (5,5-dimethyl-1,3-cyclohexanedione) is commonly used in vitro to study sulfenation of purified proteins, selectively “tagging” them, allowing monitoring by mass spectrometry. However dimedone is of little use in complex protein mixtures because selective monitoring of labeling is not possible. To address this issue, we synthesized a novel biotinylated derivative of dimedone, keeping the dione cassette required for sulfenate reactivity but adding the functionality of a biotin tag. Biotin-amido(5-methyl-5-carboxamidocyclohexane 1,3-dione) tetragol (biotin dimedone) was prepared in six steps, combining 3,5-dimethoxybenzoic acid (Birch reduction, ultimately leading to the dimedone unit with a carboxylate functionality), 1-amino-11-azido-3,6,9-trioxanodecane (a differentially substituted tetragol spacer), and biotin. We loaded biotin dimedone (0.1 mM, 30 min) into rat ventricular myocytes, treated them with H2O2 (0.1–10,000 μM, 5 min), and monitored derivatization on Western blots using streptavidin-horseradish peroxidase. There was a dose-dependent increase in labeling of multiple proteins that was maximal at 0.1 or 1 mM H2O2 and declined sharply below basal with 10 mM treatment. Cell-wide labeling was observed in fixed cells probed with avi-din-FITC using a confocal fluorescence microscope. Similar H2O2-induced labeling was observed in isolated rat hearts. Hearts loaded and subjected to hypoxia showed a striking loss of labeling, which returned when oxygen was resupplied, highlighting the protein sulfenates as oxygen sensors. Cardiac proteins that were sulfenated during oxidative stress were purified with avidin-agarose and identified by separation of tryptic digests by liquid chromatography with on-line analysis by mass spectrometry. Molecular & Cellular Proteomics 6:1473–1484, 2007.

From the ‡Department of Cardiology, Cardiovascular Division, King’s College London, The Rayne Institute, St. Thomas’ Hospital, London SE1 7EH, United Kingdom, §Department of Chemistry, Imperial College London, London SW7 2AZ, United Kingdom, and ¶Kennedy Institute of Rheumatology Division, Faculty of Medicine, Imperial College London, 1 Aspenlea Road, Hammersmith, London W6 8LH, United Kingdom

Received, February 15, 2007, and in revised form, May 30, 2007 Published, MCP Papers in Press, June 14, 2007, DOI 10.1074/mcp.M700065-MCP200

© 2007 by The American Society for Biochemistry and Molecular Biology, Inc. This paper is available on line at http://www.mcponline.org

Molecular & Cellular Proteomics 6.9 1473
Redox Modulation of Protein Sulfenic Acid Formation

Fig. 1. Reaction of a protein sulfenic acid with 5-dimethyl-1,3-cyclohexanedione, which is commonly known as dimedone. Dimedone has been widely utilized in the in vitro study of protein sulfenation but is of little use in the study of a complex mixture. This is because although it selectively conjugates with sulfenic acids it does not add any property that enables detection or purification in complex mixtures.

Fig. 2: To overcome the limitations of dimedone as a tool to study protein sulfenation in complex cell and organ systems, we functionalized dimedone by the addition of a biotin moiety. The modified dimedone retains the dione cassette required for addition to sulfenic acids but has a biotin tag, which allows detection as well as affinity purification. The synthesis protocol for the generation of biotinylated dimedone is outlined in detail here. DMF, N,N-dimethylformamide; THF, tetrahydrofuran; Ph$_3$P, triphenylphosphine.

Experimental Procedures

This investigation was performed in accordance with the Home Office “Guidance on the Operation of the Animals (Scientific Procedures) Act 1986” published by Her Majesty’s Stationery Office, London, UK.

Chemicals—These were obtained from Sigma or VWR (Poole, UK) unless stated and were of AnalaR grade or above.

Synthesis of Biotin Dimedone—Fig. 2 shows our synthesis of biotin dimedone (1), which started from the reported preparation of 1-methyl-3,5-dimethoxy-1,4-dihydrobenzoic acid (18, 19) (3), which we were able to repeat with minor modifications. It was noted that the principal contaminant was the ketone from hydrolysis of one enol ether moiety; although this may be removed chromatographically (EtOAc-hexane, 2:1, v/v), the concomitant reduction in yield led us to carry on with crude 3. The carboxylic acid was coupled with 1-azido-11-amino tetragol (20) (4) using ethyl(3-dimethylaminopropyl)carbodiimide (EDCI)† as the condensing agent. The resultant azido amide (5) was reduced to the corresponding amine (6) under Staudinger conditions, and this was condensed with biotin, again using EDCI. Finally the protected diamide (7) was unblocked under acidic conditions to give biotin dimedone (1). A full detailed description of the synthesis of biotin dimedone is provided as supplemental information.

Studying Protein Sulfenation with Biotinylated Dimedone in Isolated Rat Myocytes—Ventricular myocytes were isolated from hearts of male Wistar rats (250–300 g) using a standard collagenase digestion protocol as described previously (21). The cell suspension was main-

† The abbreviations used are: EDCI, ethyl(3-dimethylaminopropyl)carbodiimide; SNAP, S-nitroso-N-acetylpenicillamine; HNE, hydroxynonenal; NBD, 7-nitrobenzo-2-oxa-1,3-diazole; HRP, horseradish peroxidase.
Studying Protein Sulfenation with Biotinyl-Dimedone in Isolated Rat Hearts—Animals were anesthetized with sodium pentobarbitone (40 mg intraperitoneally) and injected with sodium heparin (200 IU) via the femoral vein. Hearts were rapidly excised, placed in cold (4 °C) bicarbonate buffer, cannulated, and perfused in the Langendorff mode at a constant flow of 12 ml/min/g of tissue as described previously (23) utilizing the perfusion protocols outlined in Fig. 7 with biotin dimedone loaded at 50 μM. When hypoxia was required, the bicarbonate perfusion buffer was gassed with 95%N₂, 5%CO₂, whereas 95%O₂, 5%CO₂ was used for the normoxic buffer. At the end of the perfusion protocol, hearts were frozen and stored in liquid nitrogen until further analysis. Ventricular tissue was homogenized (10 ml of buffer/g of cardiac tissue) on ice in 100 mM Tris-HCl, 100 mM maleimide, and protease inhibitors (1 Complete C tablet/50 ml; Roche Applied Science), pH 7.0, using a Polytron tissue grinder. A sample of the homogenate was reconstituted in an equal volume of 2 × SDS buffer without a reducing agent.

Protein Sulfenic Acid Detection by Western Blotting and Immunodetection—Protein samples prepared from cells or hearts were then analyzed by SDS-PAGE using the Bio-Rad mini-Protein III system. After electrophoresis samples were transferred onto PVDF membrane (GE Healthcare) using a Bio-Rad semidyeblotter. Streptavidin-horse-radish peroxidase (GE Healthcare; diluted 1:2000) was used to detect proteins containing biotin, and the ECL reagent (GE Healthcare) was used to visualize the proteins. In some studies the oxidation state of peroxiredoxin (Prx 1–4) protein was monitored with a polyclonal rabbit antibody (Lab Frontiers, Seoul, Korea) that only detects this protein in the sulfenic/sulfonic form. A polyclonal rabbit antibody (Calbiochem) that recognizes hydroxynonenal protein Michael adducts was also utilized in some studies.

Fig. 3. Detection of protein sulfenation in isolated rat ventricular myocytes. An index of protein sulfenation is provided by monitoring protein derivatization by biotin dimedone in Western blots probed with streptavidin-HRP. Experiments were undertaken to determine the optimal concentration of biotin dimedone to use, comparing each concentration tested with or without hydrogen peroxide treatment. The 75-kDa band is an endogenously biotinylated protein, which functions as a loading control. On the basis of these observations we used 100 μM biotin dimedone in all subsequent myocyte studies.

Fig. 4. A, representative Western blot probed with streptavidin-HRP demonstrating a dose-dependent response of protein sulfenation to hydrogen peroxide treatment from 0 to 1 mM. There was a pronounced transition in the signal when 10 mM hydrogen peroxide treatment was used. B, quantitation of immunoblot data from five independent experiments. C, Western immunoblot probed with an antibody that specifically detects peroxiredoxin proteins in the hyperoxidized (sulfenic and sulfonic) states. The extent of hyperoxidation detected made a marked transition at 10 mM hydrogen peroxide, consistent with the marked, concomitant loss of sulfenic acid labeling by biotin dimedone at this concentration.
Packings, Amsterdam, The Netherlands) and eluted with an acetoni-trile, 0.1% formic acid gradient. The capillary voltage was set to 3500 V. A survey scan over the m/z range 400–1300 was used to identify protonated peptides with charge states of 2, 3, or 4, which were automatically selected for data-dependent MS/MS analysis and fragmented by collision with argon. The resulting product ion spectra were transformed onto a singly charged m/z axis using a maximum entropy method (MaxEnt3, Waters), and proteins were identified by

FIG. 5. A, again hydrogen peroxide treatment of isolated rat ventricular myocytes elevated protein sulfenation as detected by biotin dimedone derivatization as monitored on Western blots probed with streptavidin-HRP. The hydrogen peroxide-induced labeling is in direct contrast to the nitric oxide donor compound SNAP, which was used because it can promote protein S-nitrosylation. Similarly diamide, which promotes disulfide bond formation, did not induce biotin dimedone adduction. B, schematic representation of the reaction of HNE with a protein to form a Michael adduct. This addition of the lipid via its double bond results in the generation of modified proteins with a carbonyl, a moiety that can potentially react with dimedone and its derivatives. C, treatment of myocytes with HNE failed to promote incorporation of biotin dimedone, although Michael adducts clearly formed during this intervention as detected with an antibody specific for these modifications that serves as a positive control. In addition, it is evident that these HNE Michael adducts did not form in myocytes treated with hydrogen peroxide, whereas this intervention clearly promoted biotin dimedone incorporation. Together these observations support the literature that suggests that dimedone is selective for addition to sulfenates because increases in protein S-nitrosylation or disulfide formation did not promote labeling.

Redox Modulation of Protein Sulfenic Acid Formation
correlation of uninterpreted spectra to entries in Swiss-Prot/TrEMBL using ProteinLynx Global Server (Version 1.1, Waters). The database was created by merging the FASTA format files of Swiss-Prot/TrEMBL and their associated splice variants (1,788,175 entries at the time of writing). No taxonomic or protein mass and pl constraints were applied. One missed cleavage per peptide was allowed, and the initial mass tolerance window was set to 100 ppm. In parallel, the spectra were also searched against the National Center for Biotechnology Information non-redundant (NCBI nr) database (3,879,234 sequences as of August 2006) using Mascot (Matrix Science). For an identification to be considered valid we required that two or more peptides independently matched the same protein sequence, that the peptide score was significant (typically greater than 55 (p < 0.05), and that manual interpretation confirmed agreement between spectra and peptide sequence. In addition Mascot searches of all spectra were performed against a randomized version of the NCBI database using the same parameters as in the main search. In no case did this search retrieve more than a single peptide, and in all instances the peptide score was below the 0.05 significance level.

Confocal Imaging of FITC-labeled Adult Ventricular Myocytes—Rat ventricular myocytes were loaded with biotin dimedone and treated with or without hydrogen peroxide as described above. Cells were then pelleted (1 min at 1000 × g) and resuspended in methanol (−20 °C) to fix the cells. The cells were repelleted, and the methanol was replaced with PBS + 100 mM maleimide + 0.5% (v/v) Triton X-100, which permeabilized the cells. Cells were blocked by rotating overnight in antibody diluent (Dako Corp.) + 100 mM maleimide. Cells were incubated with ExtrAvidin-FITC (Sigma) (1:100 in antibody diluent) for 1 h and then spin-washed four times in PBS + 0.5% Triton X-100. Cells were pelleted, resuspended in fluorescent mounting medium (Dako Corp.), plated onto glass slides, and sealed with a coverslip. Fluorescence was localized using a Zeiss LSM510 laser scanning confocal microscope (software version 1.49.44). The FITC-labeled cells were excited at a wavelength of 488 nm with an argon laser. The laser intensity and detection settings were selected to keep the autofluorescence of unlabeled myocytes to a minimum, and the same settings were maintained for analysis of treated cells.

RESULTS

Detection of Protein Sulfenic Acids in Myocytes—Fig. 3 shows protein sulfenation in primary cultures of isolated rat ventricular myocytes as detected by protein derivatization with biotin dimedone. We undertook these experiments to determine the optimal concentration of biotin dimedone to use, comparing each concentration tested with or without hydrogen peroxide treatment. The band that runs at 75 kDa is an endogenously biotinylated protein (25) that serves as a loading control. We concluded that loading cells with 0.1 mM biotin dimedone gave the best peroxide-induced signal while minimizing the amount of the compound used. On this basis 100 μM biotin dimedone was used in all subsequent studies utilizing myocytes.

Fig. 4, A and B, shows the dose response of protein sulfenation following hydrogen peroxide treatment. Clearly there was a sharp increase in protein oxidation when the peroxide concentration was elevated to 10 μM compared with the signal generated with 1 μM treatment. Sulfenation was further elevated when peroxide was increased to 100 μM, but there was no additional signal with a 1 mM challenge. However, it is notable that there was markedly less protein sulfenation observed when myocytes were treated with 10 μM peroxide. Fig. 4C shows an immunoblot probed with an antibody that detects hyperoxidized (sulfinic and sulfonic) states of the peroxiredoxin protein. The marked increase in hyperoxidation signal when cells were treated with 10 μM peroxide is consistent with the sharp decline in biotin dimedone labeling observed at this time and supports the selective reactivity of dimedone with sulfenic acids.

Fig. 5A shows again that hydrogen peroxide treatment elevated protein sulfenates. This contrasts the interventions with the nitric oxide donor compound SNAP, which was used because it promotes protein S-nitrosylation but did not cause proteins to become derivatized by biotin dimedone. Similarly diamide, which promotes disulfide bond formation, did not render proteins susceptible to biotin dimedone adduction. Similarly Fig. 5C shows that the reactive oxidized lipid hydroxynonenal (HNE) also did not promote incorporation of biotin dimedone. This lack of biotin labeling is not explained by a failure of HNE to form protein adducts. We can be sure of this because an antibody specific to HNE Michael adducts clearly detected the formation of these modifications when...
myocytes were treated with the reactive lipid. In addition, when myocytes were treated with H$_2$O$_2$, there was no formation of HNE protein Michael adducts.

Confocal Imaging of Protein Sulfenic Acids—Representative confocal images of protein sulfenation in isolated rat ventricular myocytes, as detected by biotin dinedone protein derivatization, are shown in Fig. 6. Biotin dinedone incorporation was monitored by staining fixed and permeabilized cells with ExtrAvidin-FITC. These images demonstrate that cells loaded with biotin dinedone showed global fluorescence, which was greatly enhanced by treatment with hydrogen peroxide. This labeling is consistent with the Western blot data presented in Figs. 3 and 4, which also show a peroxide-induced signal. These imaging data also confirm that intracellular protein sulfenates were detected using this analytical procedure, consistent with our anticipation that the biotin dinedone would be membrane-permeable.

Monitoring and Identification of Protein Sulfenic Acids in Isolated Heart Tissue—Fig. 8A shows a representative Western blot obtained from isolated hearts that had undergone a variety of treatments that modulate cellular redox state as outlined in the perfusion protocols shown in Fig. 7. Quantitation of these immunoblot data are shown in Fig. 8B. There was a resting level of protein sulfenation detected in aerobically perfused hearts. There was a substantial reduction below this basal state when hearts were loaded and maintained under hypoxic conditions. This hypoxia-induced decrease in protein sulfenation was rapidly reversed by reoxygenation of the heart tissue. This resupply of molecular oxygen in the perfusion buffer increased protein sulfenation such that it reinstated the level of sulfenation observed in aerobic control preparations. Hydrogen peroxide treatment was notable for its ability to substantially enhance protein sulfenation beyond that observed in aerobic controls. These proteins were affinity-purified using streptavidin and identified by analyzing tryptic digests by liquid chromatography and on-line tandem mass spectrometry. This affinity capture yielded significantly more protein when prepared from hearts treated with hydrogen peroxide compared with aerobic controls (see Fig. 9). This is consistent with the Western blot data from these studies showing enhanced incorporation of biotin dinedone in preparations administered hydrogen peroxide. Table I documents the proteins we identified that formed protein sulfenic acids in isolated rat hearts subjected to low dose hydrogen peroxide treatment.

DISCUSSION

Protein Sulfenic Acids and Cellular Redox—Healthy cells are generally found in a reduced state with the generation of both an acute and chronic cellular pro-oxidizing environment (oxidative stress) thought to be important in the pathogenesis of many diseases, including those of the cardiovascular system. However, it is now appreciated that thiol oxidation should not be exclusively equated with cellular dysfunction. The thiol reduction potential varies characteristically with cell life cycle phases such as quiescence, proliferation, confluence, differentiation, and apoptosis (26). In addition, thiol redox changes occur in healthy cells via increases in the...
activity of a variety of oxidases, which integrate proximally
with many neurohormonal signaling agents via phosphoregu-
lation (27).

There are several redox couples in cells involving equilibria
between reduced thiols and a number of their reversible oxid-
ation states. One such oxidation state is the sulfenic acid,
which is particularly labile in most proteins, often forming
transiently during enzyme catalysis or as intermediates before
oxidation to more stable states, such as the sulfonated or
S-thiolated states. However, there is evidence that some pro-
tein sulfenates are sufficiently stable to operate as post-trans-
lational regulators of function in some proteins. For example,
it is established that a number of transcription factors form
regulatory sulfenic acids, including OxyR, OhrR, and Yap1
(9, 12, 13, 28–30) Other proteins that form sulfenates in-
clude members of the NAD(P)H:disulfide reductase family,
tyrosine phosphatases, peroxidases, and methionine sulf-
oxide reductases However, most of these are bacterial pro-
teins, although some mammalian proteins have been iden-
tified (14, 31–33).

Analysis and Detection of Protein Sulfenic Acids—There
are a number of methods used to detect sulfenic acids. One
technique involves the thiol-reactive reagent 4-chloro-7-nitro-
benzo-2-oxa-1,3-diazole (NBD-Cl). NBD derivatives exhibit
different UV-visible spectra depending on whether it is ad-
ducted to reduced (–SH) or oxidized (–SOH) protein (34–36).
Therefore this method can be used to identify the presence of
sulfenic acids. However, this method is only of use with a
single purified protein and not of use in complex protein
mixtures. One strategy for detecting protein sulfenic acids in
complex biological systems involves arsenite selective reduc-
tion and labeling (14). However, the arsenite-dependent bi-
otin-switch method requires SDS denaturing conditions, and
so many sulfenates may be lost and never labeled.

5,5-Dimethyl-1,3-cyclohexanedione (dimedone) is another
compound that can selectively react with protein sulfenates,
producing a discrete adduct to cysteine sulfenates but not
other amino acids (15–17). This selective reactivity has been
utilized in the study of protein sulfenates by producing radio-
labeled versions that can be combined with electrophoresis
and autoradiography (37). Dimedone labeling can also be
combined with mass spectrometry to study protein sulfena-
tion, but again this is limited to in vitro analyses. Recently
Poole et al. (17) synthesized fluorescent derivatives of dime-
done. These compounds were used to label sulfenic sulfur,
adding a fluorescent tag to enable detection in purified pro-
teins, and confirmed selective cysteine labeling by mass
spectrometry. Although these fluorescent derivatives enable
sulfenate detection, the utility in purifying and identifying
modified proteins in cells is more limited. However, this limi-
tation might be addressed by utilizing these compounds as
part of a two-dimensional electrophoresis-DIGE proteomics
analysis. We reasoned that a related dimedone-like molecule
with a biotin label instead would allow detection as well as the
rapid and efficient affinity purification of protein sulfenates.
The synthesis strategy for a novel biotinylated dimedone mol-
ecule is outlined in detail in Fig. 2. Purification of protein

Fig. 9. Streptavidin-agarose was used to affinity purify proteins
that were sulfenated under basal conditions or after hydrogen
deroxide treatment. These proteins were resolved by SDS-PAGE
and stained with colloidal Coomassie Blue. Significantly more protein
was present in the preparation from hydrogen peroxide-treated
hearts, consistent with the Western blot data presented in Fig. 8.
Many of these proteins were identified by excising the stained bands
and producing tryptic digests that were analyzed by liquid chroma-
tography with on-line analysis by tandem mass spectrometry. The
identified proteins (numbered 1–22) are detailed in Table I.
| Band number | Identification | Matching sequences | Mascot score | Maximo decoy score |
|-------------|----------------|-------------------|--------------|--------------------|
| 1           | MYH6_RAT; myosin heavy chain, cardiac muscle α isoform | R | SNAAAAALDDKK ↓ Q | 310 | 33 |
|             |                | K | EQDTSAHLER ↓ M | | |
|             |                | K | LAEQELIETSER ↓ V | | |
|             |                | R | LQDAEEAVEAVNAK ↓ C | | |
|             |                | R | ILNPAIEPQFIDSR ↓ K | | |
|             |                | R | NTLLOAELEELEAVEQTER ↓ S | | |
| 2           | MYH6_RAT; myosin heavy chain, cardiac muscle α isoform | R | VKLLEAQR ↓ S | 126 | 29 |
|             |                | R | SNAAAAALDDKK ↓ Q | | |
|             |                | R | SLNDFTTQR ↓ A | | |
|             |                | K | DNDLLELTLAK ↓ V | | |
|             |                | R | ILNPAIEPQFIDSR ↓ K | | |
|             |                | R | NTLLOAELEELEAVEQTER ↓ S | | |
| 3           | MYH6_RAT; myosin heavy chain, cardiac muscle α isoform | R | SLSTELFK ↓ L | 329 | 32 |
|             |                | R | VKLLEAQR ↓ S | | |
|             |                | R | SNAAAAALDDKK ↓ Q | | |
|             |                | R | LQDLVDKLOKL ↓ V | | |
|             |                | R | NTLLOAELEELEAVEQTER ↓ A | | |
|             |                | K | VKLEQVDDLEGSLQEK ↓ V | | |
|             |                | R | NTLLOAELEELEAVEQTER ↓ S | | |
|             |                | K | NLQEEISDLTEQLGEGGKNVHELEK ↓ I | | |
| 4           | MYH6_RAT; myosin heavy chain, cardiac muscle α isoform | R | SLSTELFK ↓ L | 447 | 32 |
|             |                | R | VKLLEAQR ↓ S | | |
|             |                | R | SNAAAAALDDKK ↓ Q | | |
|             |                | R | LQDLVDKLOKL ↓ V | | |
|             |                | R | NTLLOAELEELEAVEQTER ↓ A | | |
|             |                | K | VKLEQVDDLEGSLQEK ↓ V | | |
|             |                | R | NTLLOAELEELEAVEQTER ↓ S | | |
|             |                | K | NLQEEISDLTEQLGEGGKNVHELEK ↓ I | | |
| 5           | MYH6_RAT; myosin heavy chain, cardiac muscle α isoform | R | SNAAAAALDDKK ↓ Q | 322 | 27 |
|             |                | R | TLEDQANEYR ↓ V | | |
|             |                | R | NTLLOAELEELEAVEQTER ↓ A | | |
|             |                | R | LQDAEEAVEAVNAK ↓ C | | |
|             |                | R | IEEELLEELEAVEQTER ↓ T | | |
| 6           | g|29727; cardiacβ myosin heavy chain | K | LASADIYLYKE ↓ S | 32 | 21 |
| 7           | ACTN2_MOUSE; α-actinin 2 | R | HRPDLDYSS ↓ L | 95 | 16 |
|             |                | R | FAIQDISVEETSAK ↓ E | | |
|             |                | K | LVSGIAEVDGNVK ↓ M | | |
| 8           | g|29727; cardiac β myosin heavy chain | K | LASADIYLYKE ↓ S | 66 | 25 |
|             | gi|6755256; muscle glycogen phosphorylase | R | YEFGCNQ ↓ I | 58 | |
|             |                | R | QIEQLSGFFSPK ↓ Q | | |
| 9           | MYH7_RAT; myosin heavy chain, cardiac muscle β isoform | K | VNGVEYVT ↓ G | 118 | 31 |
|             | gi|10637996; Mitochondrial aconitase | K | LASADIYLYKE ↓ S | 63 | |
|             |                | K | SQFTIPSGSEQR ↓ A | | |
|             |                | R | NAVTDFGPVPDTR ↓ Y | | |
| 10          | ECHA_RAT; trifunctional enzyme α subunit | K | KLDALETVQGAATLDAEVGIDVAQHVAEDLGK ↓ A | 62 | 20 |
| 11          | ODP2_RAT; dihydrolipoamide-residue acetyltransferase component of pyruvate dehydrogenase complex | K | GLETIASDVVSLASK ↓ A | 48 | 23 |
|             | gi|219524; cardiac α myosin heavy chain | R | SNAAAAALDDKK ↓ Q | 44 | |
|             | gi|6754774; myosin heavy polypeptide 7, cardiac muscle, β | R | SNAAAAALDDKK ↓ Q | 76 | 29 |
|             | gi|109466092; 3-oxoacid CoA transferase 1 | K | GTLEQIOQANPALEAGNNAK ↓ T | 55 | 30 |
|              | ATPB_RAT; ATP synthase β chain | R | FGAGSEVSALLGR ↓ I | 55 | 30 |
|              |                | K | VLDGAPIKIVPGEVTLGR ↓ I | | |

TABLE I

Protein sulfenic acids formed in isolated hearts treated with hydrogen peroxide
We initially used primary cultures of isolated rat smooth muscle cells and exposed them to hydrogen peroxide concentration was elevated from 1 to 10 mM. This is likely explained by overoxidation of sulfenates to sulfinic or sulfonic acid at a reaction rate that is faster than the reaction of sulfenates with hydrogen peroxide. These sulfenations are consistent with the ability of hydrogen peroxide to oxidize protein thiols often en route to further oxidative modifications such as S-glutathiolation being a principal end product in cells. We can conclude that these two widespread and common cysteine oxidation states (S-nitrosylation, whereas dia-เสนเด) do not are known to have redox cysteines susceptible to sulfenic acid formation as well as subsequent transition to higher oxidation states (5–7, 12), and so it is useful to compare their oxidation state with the data collected utilizing biotin dimedone. In contrast to hydrogen peroxide, neither of the thiol oxidants SNAP or diamide promoted protein sulfenation as evidenced by biotin dimedone incorporation. The nitric oxide donor SNAP promotes protein S-nitrosylation, whereas diamide is efficient in promoting disulfide bond formation with S-glutathiolation being a principal end product in cells. We can conclude that these two widespread and common cysteine oxidation states (S-nitrosylated and disulfide) do not react with biotin dimedone, further supporting the selectivity of this reagent for sulfenates. Another common post-translational oxidative modification of proteins involves carbonyl formation. These events commonly occur in systems where reactive lipid aldehydes, such as HNE, accumulate (38). HNE adducts undergo addition reactions with proteins involving a Michael addition to the lipid double bond (Fig. 5B). This mech-

| Band number | Identification                  | Matching sequences | Mascot score | Maxim decoy score |
|-------------|--------------------------------|--------------------|-------------|------------------|
| 14          | ATPB_MOUSE; ATP synthase β chain | R | VVDALGNAIDGK | G | 208 | 20 |
|             |                                | R | EAYPGDFVYHSLR | L | 197 | |
|             |                                | R | ILGADTSVDELTEG | V | 155 | 24 |
|             |                                | R | TGAIVDYPYGDELLGRVDVLGNAIDGKPVGSK | I | 128 | |
|             | gi50677; desmin                 | K | VPPPLPOQGR | K | 96  | 23 |
|             | ACTC_MOUSE; actin, α cardiac    | R | SEVELQVIDVGNLVDCEK | K | 77  | 26 |
|             |                                | R | AVFPSIVGR | A | 89  | 28 |
|             |                                | K | DSVGYGEAQSKR | G | 69  | 17 |
|             |                                | K | SYELPGPVQTIGNER | F | 71  | 21 |
| 15          | KCRS_MOUSE; creatine kinase     | R | DAEIDGPVDSDKPKPSR | L | 72  | 33 |
|             |                                | K | DLNELQTLIEAHFENR | K | 63  | 29 |
|             |                                | K | AKELWNSIHINLEAFQDLOE | F | 58  | 17 |
|             |                                | K | SIDDLEDSELYAQ | K | 44  | 17 |
|             |                                | K | IFGVTTLDIVR | A | 35  | |
| 16          | TNNT2_RAT; troponin T, cardiac muscle | R | KLIIEESDLER | A | 20  | 12 |
|             |                                | K | SIDLEDELYAQK | L | 19  | 12 |
|             |                                | K | IFGVTTLDIVR | A | 18  | 12 |
|             |                                | K | ETETCTYFSTPLLGG | K | 16  | 12 |
|             | gi1200100; malate dehydrogenase | K | IQEAGTEVVK | A | 14  | 12 |
|             | gi71620; actin β                | K | VAVLGASGGGIGQPSL | L | 12  | 12 |
|             |                                | K | IFPVETLVEAIAQCEK | I | 11  | 12 |
|             | gi56023; cardiac troponin I     | K | NITEIATLTKQ | I | 10  | 12 |
|             | gi20071222; NADH dehydrogenase Fe-S protein 3 | K | NITEIATLTKIYDLR | G | 9  | |
|             |                                | K | NITEIATLTKIYDLR | G | 8  | |
|             |                                | V | VDFQETK | V | 7  | |
|             | gi6981240; myosin, light polypeptide 3 | K | ITAEPCQAEVFQ | L | 6  | |
|             |                                | K | VDFQETK | V | 5  | |
|             | gi56023; cardiac troponin I     | K | NITEIATLTKIYDLR | G | 4  | |
|             | gi20071222; NADH dehydrogenase Fe-S protein 3 | K | NITEIATLTKIYDLR | G | 3  | |
|             |                                | K | NITEIATLTKIYDLR | G | 2  | |
|             | gi56023; cardiac troponin I     | K | NITEIATLTKIYDLR | G | 1  | |
|             | gi20071222; NADH dehydrogenase Fe-S protein 3 | K | NITEIATLTKIYDLR | G | 0  | |
|             |                                | V | VDFQETK | V | 0  | |
|             | gi56023; cardiac troponin I     | K | NITEIATLTKIYDLR | G | 0  | |
|             | gi20071222; NADH dehydrogenase Fe-S protein 3 | K | NITEIATLTKIYDLR | G | 0  | |
|             |                                | K | NITEIATLTKIYDLR | G | 0  | |
|             | gi56023; cardiac troponin I     | K | NITEIATLTKIYDLR | G | 0  | |
|             | gi20071222; NADH dehydrogenase Fe-S protein 3 | K | NITEIATLTKIYDLR | G | 0  | |

**TABLE I—continued**

* Arrows indicate tryptic cleavage sites.
* Individual ion scores >57 indicate identity or extensive homology (p < 0.05).
* Maxim decoy score is the maximum score obtained by searching the same spectra against the randomized database using the same parameters.
anism leads to protein carbonyl formation and is thought to be the dominant reaction product, forming more readily than adducts involving conjugation with primary amines that can also react with the carbonyl group to form Schiff bases (39). To investigate the possibility of biotin dimedone incorporation via reaction with carbonyls, we assessed whether this happens in myocytes treated with HNE. Indeed we could not detect any such modifications, even at 100 μM, that directly contrasts efficient biotin dimedone labeling with 10 μM H₂O₂. It was possible that the HNE was not forming protein conjugations in our model system, particularly the Michael adducts required to generate protein carbonyls. However, this is not the case as an antibody specific for Michael adducts gave robust labeling in myocytes treated with HNE. It is notable, however, that the same antibody did not detect HNE protein modifications in response to H₂O₂ treatment. We had anticipated that H₂O₂ treatment would likely generate Michael adducts in our model system; but regardless of this, incorporation of biotin dimedone did not occur when carbonyls were incorporated after direct treatment with HNE. The reaction of carbonyls with dimedone has been studied and generates several reaction products, although the dominant cyclized product is derived from two dimedone molecules per carbonyl and requires ammonia (40). It is possible that the presence of the biotin tag somehow interferes with this reaction chemistry and also that it does not occur for protein carbonyls in the same way as it does for aliphatic carbonyl compounds. So although it is possible that biotin dimedone may label proteins via chemistry involving carbonyl modifications, such as by hydroxynonenal, this does not appear to be an issue in these studies. We concede that this is not a definitive demonstration that carbonyl formation absolutely does not facilitate biotin dimedone labeling. However, detection of such events would be of potential interest themselves, although if this was a common event it would complicate interpretation of labeling simply as sulfenation. However, close scrutiny of most labeling reagents used for “specific” labeling of oxidized biomolecules generally will unveil potential alternate reaction chemistries. Ultimately the detection of proteins that are labeled in response to oxidative stress is likely of general interest even if the proteins did not involve sulfenic acids. Once potential sulfenation targets are identified, proteins can be individually analyzed to corroborate (or not) whether they were indeed sulfenated.

**Studying Protein Sulfenic Acids in Isolated Hearts**—Our studies also showed that biotin dimedone can be used to study protein sulfenation in isolated hearts. These studies illustrate that the extent of protein sulfenation was modulated both by the oxygen tension and by hydrogen peroxide. There was a basal level of oxidation in control preparations, consistent with a dependence on molecular oxygen. A resting level of protein sulfenation is surmised because hearts subjected to hypoxia show a striking loss of labeling, which rapidly recovered on reoxygenation. Whether sulfenation involves direct oxidation by molecular oxygen or occurs via secondary reactive oxygen species formed at enhanced levels with higher oxygen tension cannot be firmly concluded. As sulfoxidation of proteins can occur by air oxidation, it is perhaps reasonable to conclude that at least some protein sulfenates form directly from thiols reacting directly with molecular oxygen. The decrease in thiol oxidation state during oxygen deprivation is consistent with the observations in a recent study in yeast (27). Although in that study, the ability of hydrogen peroxide to elicit thiol oxidations was perhaps less marked with only a few specific proteins identified as selectively peroxide-responsive.

*Proteins Identified That Form Sulfenic Acids*—In this study a diverse range of proteins was identified that formed sulfenic acids (see Table I). These protein sulfenates are generally in line with those identified in a previous study utilizing arsenite selective reduction to label and subsequently identify proteins altered in this way. It is interesting to speculate that these sulfenic acid modifications may be important regulators of protein function, operating as a molecular switch that is redox-sensitive. In this scenario, the sulfenate might be a post-translational oxidative modification that alters function especially when the modified cysteine is catalytically crucial for enzymatic activity or is part of a reaction cycle. Alternately the redox state of cysteines could influence protein-protein interactions, and these modified cysteines could serve as mediators of signaling in that way. Although we were able to identify proteins that form sulfenates by their reactivity with a biotinylated derivative of dimedone, it remains possible that sulfenates are not important from a post-translational regulatory standpoint. Instead we may have trapped protein sulfenates as they were transiently formed. For example, perhaps only a small, stoichiometrically irrelevant proportion of proteins form the sulfenate at any given time. Very unstable, short lived modifications are unlikely to operate directly as a regulatory modification. The instability of sulfenates relates to their reactivity with other abundant cellular components such as thiols (resulting in the formation of more stable disulfides) or oxidants that may induce hyperoxidation to the sulfinic or sulfonic state. With regard to sulfenates being intermediates prior to formation of other redox states, it is interesting that many of the sulfenated proteins detected here and in our previous study (14) have also been shown to be susceptible to other forms of cysteine-targeted oxidation, such as sulfide formation or S-nitrosylation (22, 41–44). However, it remains possible that some protein sulfenic acids are relatively stable by being less susceptible to subsequent redox alteration once formed and consequently might serve post-translational regulatory roles. The identification of redox-modified proteins, as in this study, may in some cases identify protein not previously known to be directly regulated by its sulfoxidation redox state. For example, our previous proteomics screens for proteins that form disulfides (41, 43) led to subsequent studies demonstrating the cysteine redox regulation of heat shock protein 27 (45) and Type I protein kinase A (46).

The proteins we have shown here to form sulfenates are on
the whole highly expressed in cardiac tissue. This is a general problem in redox proteomics studies such that most screening strategies have a bias for the detection of abundant proteins. For example, the non-stoichiometric (e.g. 1%) modification of a very abundant protein will generate a signal on a stained protein gel similar to that of a protein with stoichiometric additions that is a 100-fold less abundant. This rough calculation compares proteins of similar molecular weights and Coomassie (or other stain) binding affinities. This problem relates to the very large variations in the relative abundance of proteins and is a common scenario in proteomics (1). However, quantitative measures or strategies that normalize the dynamic range of protein abundance can be used to address this issue; but in their absence many proteomics strategies preferentially identify the same dominant species. Consequently interesting and important modifications in the subproteome, such as signaling components, are likely missed with the identifications dominated by highly abundant metabolic and mitochondrial proteins. Another issue that we encountered is the presence of proteins (namely the ATP synthase β subunit and troponin T) in affinity-purified preparations that in fact have no cysteine residues and so cannot form sulfenates. We had the same finding in a previous study (42) and reasoned that the ATP synthase β was likely present because it tightly binds the redox-active α subunit of the ATP synthase, which was then also present. However, the α subunit was not identified in the present work, and so we cannot provide a full explanation for the presence of the β subunit; although perhaps the α subunit was actually there but not identified. Similarly we cannot explain the presence of troponin T; but again this can be explained by it co-purification with myofilament proteins that do indeed have redox-active thiols. For example, tropomyosin and actin, which directly bind troponin T, were present and so likely explain its presence. Tropomyosin and actin have redox-active cysteines (47, 48), consistent with their formation of sulfenic acids as shown in this study.

In conclusion we showed that biotin dimedone is a useful novel reagent in the study of protein sulfenation, although there are some caveats in its use and also in simply interpreting all streptavidin affinity-purified proteins as sulfenates. However, overall we successfully utilized biotin dimedone to study oxidative stress in cardiac tissue and demonstrated that protein sulfenates formed under pro-oxidizing conditions. It is perhaps particularly salient that the sulfenates not only formed in response to elevations in cellular hydrogen peroxide but also that their abundance was modulated by the tension of molecular oxygen. This highlights protein sulfenation as a mechanism of oxygen sensing and also illustrates that many proteins can directly respond to alterations in oxygen availability in tissues. This leads to the possibility that proteins that respond to variations in oxygen by varying their sulfenic acid status may be functionally regulated in this way, a prospect that warrants further study.

* This work was supported by grants from The Biotechnology and Biological Sciences Research Council and The Medical Research Council. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

REFERENCES

1. Eaton, P. (2006) Protein thiol oxidation in health and disease: techniques for measuring disulfides and related modifications in complex protein mixtures. *Free Radic. Biol. Med.* 40, 1889–1899

2. Stamler, J. S., Lamas, S., and Fang, F. F. (2001) Nitrosylation: the prototypic redox-based signaling mechanism. *Cell* 106, 675–683

3. Mailis, R. J., Hamann, M. J., Zhao, W., Zhang, T., Hendrich, S., and Thomas, J. A. (2002) Irreversible thiol oxidation in carbonic anhydrase III. Protection by S-glutathiolation and detection in aging rats. *Biol. Chem.* 383, 649–662

4. Biteau, B., Labarre, J., and Toledano, M. B. (2003) ATP-dependent reduction of cysteine–sulphinic acid by S. cerevisiae sulphiredoxin. *Nature* 425, 980–984

5. Woo, H. A., Jeong, W., Chang, T. S., Park, K. J., Park, S. J., Yang, J. S., and Rhee, S. G. (2005) Reduction of cysteine sulfenic acid by sulfiredoxin is specific to 2-Cys peroxiredoxins. *J. Biol. Chem.* 280, 3125–3128

6. Chang, T. S., Jeong, W., Woo, H. A., Lee, S. M., Park, S., and Rhee, S. G. (2004) Characterization of mammalian sulfiredoxin and its reactivation of hyperoxidized peroxiredoxin through reduction of cysteine sulfenic acid in the active site to cysteine. *J. Biol. Chem.* 279, 50994–51001

7. Woo, H. A., Chae, H. Z., Hwang, S. C., Yang, K. S., Kang, S. W., Kim, K., and Rhee, S. G. (2003) Reversing the inactivation of peroxiredoxins caused by cysteine sulfenic acid formation. *Science* 300, 653–656

8. Budanov, A. V., Sablina, A. A., Feinstein, E., Koonin, E. V., and Chumakov, P. M. (2004) Regeneration of peroxiredoxins by p53-regulated sestrins, a homologue of bacterial AhpD. *Science* 304, 596–600

9. Claiborne, A., Yeh, J. I., Mallett, T. C., Luba, J., Crane, E. J., Charrier, V., and Parsonage, D. (1999) Protein-sulfenic acids: diverse roles for an unlikely player in enzyme catalysis and redox regulation. *Biochemistry* 38, 15407–15416

10. Paget, M. S. B., and Buttner, M. J. (2003) Thiol-based regulatory switches. *Annu. Rev. Genet.* 37, 91–121

11. Green, J., and Paget, M. S. (2004) Bacterial redox sensors. *Nat. Rev. Microbiol.* 2, 954–966

12. Poole, L. B., Karpilus, P. A., and Claiborne, A. (2004) Protein sulfenic acids in redox signaling. *Annu. Rev. Pharmacol. Toxicol.* 44, 325–347

13. Claiborne, A., Miller, H., Parsinage, D., and Ross, R. P. (1999) Protein-sulfenic acid stabilization and function in enzyme catalysis and gene regulation. *FASEB J.* 7, 1483–1490

14. Saurin, A. T., Neubert, H., Brennan, J. P., and Eaton, P. (2004) Widespread sulfenic acid formation in tissues in response to hydrogen peroxide. *Proc. Natl. Acad. Sci. U. S. A.* 101, 17982–17987

15. Carballal, S., Radi, R., Kirk, M. C., Barnes, S., Freeman, B. A., and Alvarez, B. (2003) Sulfenic acid formation in human serum albumin by hydrogen peroxide and peroxynitrite. *Biochemistry* 42, 9906–9914

16. Raftery, M. J., Yang, Z., Valenzuela, S. M., and Geisz, C. L. (2001) Novel intra- and inter-molecular sulfonamide bonds in S100A8 produced by hypochlorite oxidation. *J. Biol. Chem.* 276, 33393–33401

17. Poole, L. B., Zeng, B. B., Knaggs, S. A., Yakubu, M., and King, S. B. (2005) Synthesis of chemical probes to map sulfenic acid modifications on proteins. *Bioconjug. Chem.* 16, 1624–1628

18. Gopalan, A., and Magnus, P. (1984) Total synthesis of (±)-linderalactone, (±)-isolinderalactone, and (±)-neolinderalactone, germacrane furano-

sesquiterpenes. *J. Org. Chem.* 49, 2317–2321

19. Chapman, O. L., and Fitton, P. (1963) A general synthesis of the troponoid system based on solvolysis of 1,4-dihydrobenzyl tosylates. *J. Org. Chem.* 38, 41–47

20. Schwabacher, A. W., Lane, J. W., and Schieser, M. W. (1998) Desymmetry- tion reactions: efficient preparation of unsymmetrically substituted
Redox Modulation of Protein Sulfenic Acid Formation

21. James, A. F., Ramsey, J. E., Reynolds, A. M., Hendry, B. M., and Shattock, M. J. (2001) Effects of endothelin-1 on K+ currents from rat ventricular myocytes. *Biochem. Biophys. Res. Commun.* **284**, 1048–1055

22. Brennan, J. P., Miller, J. I. A., Fuller, W., Wait, R., Begum, S., Dunn, M. J., and Eaton, P. (2006) The utility of N,N-biotinylation glutathione disulfide in the study of protein S-glutathiolation. *Mol. Cell. Proteomics* **5**, 215–225

23. Eaton, P., Bell, R. M., Cave, A. C., and Shattock, M. J. (2005) Ischemic preconditioning: a potential role for protein S-thiolation? *Antioxid. Redox Signal.* **7**, 882–888

24. Wait, R., Gianazza, E., Eberini, I., Sironi, L., Dunn, M. J., Gemenie, M., and Miller, I. (2001) Proteins of rat serum, urine, and cerebrospinal fluid: VI. Further protein identifications and interstrain comparison. *Electrophoresis* **22**, 3043–3052

25. Oei, J., and Robinson, B. H. (1985) Simultaneous preparation of the three biotin-containing mitochondrial carboxylases from rat liver. *Biochim. Biophys. Acta* **840**, 1–5

26. Schafer, F. Q., and Buettner, G. R. (2001) Redox environment of the cell viewed through the redox state of the glutathione disulfide/glutathione couple. *Free Radic. Biol. Med.* **30**, 1191–1212

27. Le Moan, N., Clement, G., Le Maout, S., Tacnet, F., and Toledano, M. B. (2006) The Saccharomyces cerevisiae proteome of oxidized protein thiols: contrasted functions for the thioedoxin and glutathione pathways. *J. Biol. Chem.* **281**, 10420–10430

28. Choi, H. J., Kim, S. J., Mukhopadhyay, P., Cho, S., Woo, J. R., Storz, G., and Ryu, S. E. (2001) Structural basis of the redox switch in the OxyR transcription factor. *Cell* **105**, 103–113

29. Denu, J. M., and Tanner, K. G. (1998) Specific and reversible inactivation of protein tyrosine phosphatases by hydrogen peroxide: evidence for a sulfenic acid intermediate and implications for redox regulation. *Biochemistry* **37**, 5633–5642

30. Elaibane, A., Mallet, T. C., Yeh, J. I., Luba, J., and Parsonage, D. (2001) Structural, redox, and mechanistic parameters for cysteine-sulfenic acid function in catalysis and regulation. *Adv. Protein Chem.* **58**, 215–276

31. Allison, W. S. (1976) Formation and reactions of sulfenic acids in proteins. *Acc. Chem. Res.* **9**, 293–299

32. Allison, W., and Benitez, L. V. (1972) An adenosine triphosphate exchange catalyzed by a soluble enzyme couple inhibited by uncouplers of oxidative phosphorylation. *Proc. Natl. Acad. Sci. U. S. A.* **69**, 3004–3008

33. Storz, G., Tartaglia, L. A., and Ames, B. N. (1990) Transcriptional regulation of oxidative stress-inducible genes: direct activation by oxidation. *Science* **248**, 189–194

34. Ellis, H. R., and Poole, L. B. (1997) Novel application of 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole to identify cysteine sulfenic acid in the AhpC component of alkyl hydroperoxide reductase. *Biochemistry* **36**, 15013–15018

35. Kim, S. O., Merchant, K., Nudelman, R., Beyer Jr., J. F., Keng, T., DeAngelis, J., Hausladen, A., and Stamler, J. S. (2002) OxyR: a molecular code for redox-related signaling. *Cell* **109**, 383–396

36. Poole, L. B., and Ellis, H. R. (2002) Identification of cysteine sulfenic acid in AhpC of alkyl hydroperoxide reductase. *Methods Enzymol.* **348**, 122–136

37. Benitez, L. V., and Allison, W. S. (1974) The inactivation of the acyl phosphatase activity catalyzed by the sulfenic acid form of glyceraldehyde 3-phosphate dehydrogenase by dimedone and olefins. *J. Biol. Chem.* **249**, 8234–8243

38. Esterbauer, H., Schaur, R. J., and Zollner, H. (1991) Chemistry and biochemistry of 4-hydroxynonenal, malonaldehyde and related aldehydes. *Free Radic. Biol. Med.* **11**, 81–128

39. Schaur, R. J. (2003) Basic aspects of the biochemical reactivity of 4-hydroxynonenal. *Mol. Asp. Med.* **24**, 149–159

40. Zurek, G., and Karst, U. (1999) Liquid chromatography-mass spectrometry method for the determination of aldehydes derivatized by the Hantzsch reaction. *J. Chromatogr. A* **864**, 191–197

41. Eaton, P., Byers, H. L., Leeds, N., Ward, M. A., and Shattock, M. J. (2002) Detection, quantitation, purification and identification of cardiac proteins S-thiolated during ischemia and reperfusion. *J. Biol. Chem.* **277**, 9806–9811

42. Brennan, J. P., Bardswell, S. C., Burgoyne, J. R., Fuller, W., Schroder, E., Leung, K. Y., Ward, M. A., Pratt, J., and Shattock, M. J. (2003) Reversible cysteine-targeted oxidation of proteins during renal oxidative stress. *J. Am. Soc. Nephrol.* **14**, S290–S296

43. Brennan, J. P., Wait, R., Begum, S., Bell, R. J., Dunn, M. J., and Eaton, P. (2004) Detection and mapping of widespread intermolecular protein disulfide formation during cardiac oxidative stress using proteomics with diagonal electrophoresis. *J. Biol. Chem.* **279**, 41352–41360

44. Kunczewicz, T., Sheta, E. A., Goldknopf, I. L., and Kone, B. C. (2003) Proteomic analysis of S-nitrosylated proteins in mesangial cells. *Mol. Cell. Proteomics* **2**, 156–163

45. Eaton, P., Fuller, W., and Shattock, M. J. (2002) S-Thiolation of HSP27 regulates its multimeric aggregate size independently of phosphorylation. *J. Biol. Chem.* **277**, 21189–21196

46. Brennan, J. P., Bardswell, S. C., Burgoyne, J. R., Fuller, W., Schroder, E., Wait, R., Begum, S., Kentish, J. C., and Eaton, P. (2006) Oxidant-induced activation of type I protein kinase A is mediated by RI subunit interprotein disulfide bond formation. *J. Biol. Chem.* **281**, 21827–21836

47. Wang, J., Boja, E., Tan, W. H., Tekle, E., Fales, H. M., English, S., Mieyal, J. J., and Chock, P. B. (2001) Reversible glutathionylation regulates its multimeric aggregate size independently of phosphorylation. *J. Biol. Chem.* **277**, 9806–9811

48. Frattetti, M., Demoli, H., Pujpe, M., Casagrande, S., Eberini, I., Salomina, M., Bonetto, V., Mengozzi, M., Duffieux, F., Miclet, E., Bachi, A., Vandekerckhove, J., Gianazza, E., and Ghezzi, P. (2002) Identification by redox proteomics of glutathionylated proteins in oxidatively stressed human T lymphocytes. *Proc. Natl. Acad. Sci. U. S. A.* **99**, 3505–3510