The Utility of \( N,N\)-Biotinyl Glutathione Disulfide in the Study of Protein S-Glutathiolation*\[S\]

Jonathan P. Brennan‡, Jonathan I. A. Miller‡, William Fuller§, Robin Wait¶, Shajna Begum¶, Michael J. Dunn||, and Philip Eaton‡**

Glutathione disulfide (GSSG) accumulates in cells under an increased oxidant load, which occurs during neurohormonal or metabolic stimulation as well as in many disease states. Elevated GSSG promotes protein S-glutathiolation, a reversible post-translational modification, which can directly alter or regulate protein function. We developed novel strategies for the study of protein S-glutathiolation that involved the simple synthesis of \( N,N\)-biotinyl glutathione disulfide (biotin-GSSG). Biotin-GSSG treatment of cells mimics a defined component of oxidative stress, namely a shift in the glutathione redox couple to the oxidized disulfide state. This induces widespread protein S-glutathiolation, which was detected on non-reducing Western blots probed with streptavidin-horseradish peroxidase and imaged using confocal fluorescence microscopy and ExtrAvidin-FITC. S-Glutathiolated proteins were purified using streptavidin-agarose and identified using proteomic methods. We conclude that biotin-GSSG is a useful tool in the investigation of protein S-glutathiolation and offers significant advantages over conventional methods or antibody-based strategies. These novel approaches may find widespread utility in the study of disease or redox signaling models where GSSG accumulation occurs. 

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Oxidative stress has been implicated in the pathogenesis of most major diseases (1). In addition, the role of redox mechanisms in regulatory processes and everyday function of cells and tissues is beginning to be recognized, although the details of these events are not fully elucidated.

Some protein thiols are prone to oxidative modification through a variety of mechanisms, which may involve reactive oxygen species, nitric oxide, or glutathione. Reversible oxidative alterations of cysteines, such as S-glutathiolation, provide a mechanism whereby protein function may be altered in a regulated way akin to the well established phosphoregulation of proteins (2). Not all protein thiols are equally susceptible to oxidation, and their reactivity will depend on a variety of factors including the species of oxidant, the molecular accessibility of the thiol, and the chemical environment in which the reaction will take place. Many redox-active cysteines have a lower than average \( \text{pK}_\text{a} \) which potentiates the reactivity of the thiol group at physiological pH values (2, 3).

A characteristic hallmark of many diseases is a decrease in the GSH:GSSG\[1\] ratio. For example a decrease in this redox couple is associated with neurodegenerative diseases (4), human immunodeficiency virus infection and AIDS (5), cystic fibrosis (6), cancer (7), tissue ischemia (8), and aging (9), indicating the presence of an oxidative stress. When the GSSG accumulates in cells, it can undergo disulfide exchange reactions with proteinaceous thiols, leading to their S-glutathiolation, which can impact on function as highlighted above. At this time the full extent that protein S-glutathiolation may play in disease progression, redox signaling, and other pro-oxidative events remains unclear and may be explained in part by the lack of accessible methodologies for studying these events.

Here we report novel methodology for the study of protein S-glutathiolation in which we biotinylated GSSG to generate \( N,N\)-biotinyl glutathione disulfide (see Fig. 1). We refer to this compound as biotin-GSSG for brevity despite the fact the molecule contains two biotin moieties. We used biotin-GSSG as a probe for proteins that are prone to S-glutathiolation. The addition of biotin-GSSG to model systems mimics a defined component of a cell, tissue, or organ under oxidative stress, namely an increase in cellular GSSG. This increase in GSSG leads to thiol-disulfide exchange reactions, promoting protein S-glutathiolation. A benefit of this approach is that proteins that become S-glutathiolated also carry a biotin tag, which allows their detection on Western blots, cellular localization by fluorescence microscopy, and their purification using appropriate avidin-based techniques. The proteins that become labeled represent a discrete pool of proteins that are particularly susceptible to S-glutathiolation.

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\[1\] The abbreviations used are: GSSG, glutathione disulfide; biotin-GSSG, \( N,N\)-biotinyl glutathione disulfide; HRP, horseradish peroxidase.
is attached to the amine group of L-glutamates on the glutathione disulfide molecule. An extended spacer arm minimizes any steric hindrance between the biotin tag and GSSG molecule.

**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.

**Biotinylation of GSSG**—A water-soluble biotinylation reagent, sulfosuccinimidyl-6-(biotinamido)hexanoate (Merck Biosciences Ltd., Nottingham, UK), was used to couple biotin to the primary amino groups of glutathione disulfide (Sigma) under mild alkaline conditions. Specifically, the biotinylation reagent (111.4 mg) was added to GSSG (61.2 mg) in H2O (1.8 ml), pH was adjusted to 7.2 with NaOH, and the mixture was left to derivatize for 1 h at room temperature. This was made up to a final volume of 2 ml with 1 M Tris-HCl, pH 7.2 to quench the reaction. HPLC analysis using an APEX, 5-μm ODS column (Jones Chromatography, Hengoed, UK) with detection at λmax (190–400 nm) using a diode array detector showed that this reaction resulted in a single dominant product. MALDI-TOF mass spectra, acquired on an Autoflex mass spectrometer (Bruker Daltonics), confirmed this product has a mass of 1290.85, consistent with the given mass. MALDI-TOF mass spectra, acquired on an Autoflex mass spectrometer (Bruker Daltonics), confirmed this product has a mass of 1290.85, consistent with the given mass.

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leimide. Maleimide was present to block all free SH groups and prevent thiol-disulfide exchange or thiol oxidation during preparation. In a separate series of experiments, different aliquots of heart homogenate were treated either with either 1) water as a control, 2) GSSG as an additional control, 3) biotin-GSSG (5 mM), or 4) GSSG (20 mM) for 10 min alone or prior to 10-min treatment with biotin-GSSG (5 mM).

As before, the treated homogenates were reconstituted in SDS sample buffer for Western analysis to assess protein S-glutathiolation (measured on Westerns probed with streptavidin-HRP). We focused on heart tissue in these studies as much of our research has a particular focus on cardiac protein oxidation.

**Ventricular Myocyte Preparation**—Ventricular myocytes were isolated from hearts of male Wistar rats (250–300 g) using a standard collagenase digestion protocol as described previously (10). The cell suspension was maintained in modified Tyrode’s solution containing 130 mM NaCl, 5.4 mM KCl, 1.4 mM MgCl2, 0.4 mM Na2HPO4, 4.2 mM HEPES, 10 mM glucose, 20 mM taurine, 10 mM creatine, and 1.0 mM CaCl2 at room temperature for 3 h prior to use.

**Myocyte Treatment with GSSG and Diamide and Subcellular Fractionation—Glutathione disulfide (disodium salt) and diamide (CH2)=NCON=NCON(CH2)=N (Sigma, product code D 3648) stocks were made up in modified Tyrode’s solution. The cells isolated from one heart preparation were divided into three equal aliquots and treated for 10 min with GSSG (20 mM), diamide (0.5 mM), or modified Tyrode’s solution (control). Following treatment cells were pelleted at 500 x g for 0.5 min, and the supernatant was removed. Cells were resuspended in cell lysis buffer comprising 50 mM Tris-HCl, pH 7.5, 5 mM EGTA, 2 mM EDTA, 100 mM NaF, 0.05% digitonin, and 100 mM maleimide and incubated on ice for 5 min with frequent vortexing. The samples were then centrifuged at 10,000 x g for 2 min, and the supernatant was removed and kept (cytosolic fraction). The pellet was washed in lysis buffer and then resuspended in lysis buffer supplemented with 1% Triton X-100. The samples were centrifuged again, and the supernatant was kept (membrane fraction). The Triton X-100-insoluble pellet (myofilament fraction) was washed and then resuspended in lysis buffer. Fractions were reconstituted in SDS sample buffer, free of reducing agents, containing 100 mM maleimide.

**Myocyte Treatment with Biotin-GSSG**—Myocytes from one heart were divided into equal aliquots and treated for 10 min with a range of concentrations of biotin-GSSG (0.01–10 mM). Following treatment cells were pelleted at 500 x g for 0.5 min, and the supernatant was removed. Cells were resuspended in modified Tyrode’s solution and reconstituted in SDS sample buffer, free of reducing agents, containing 100 mM maleimide. In separate experiments myocytes from one heart were divided into equal aliquots, as before, and treated for 10 min with a single concentration of biotin-GSSG (5 mM) or an equal volume of water (control). After treatment, samples underwent subcellular fractionation as described above and were reconstituted in SDS sample buffer, free of reducing agents, containing 100 mM maleimide.

**SDS-PAGE-Western Blotting**—SDS-polyacrylamide gel electrophoresis was carried out using the Bio-Rad Mini Protein II system. 10 μl of tissue samples (heart, lung, kidney, brain, skeletal muscle, and liver), unfractionated myocyte samples, or myocyte subcellular fractions (cytosolic, membrane, and myofilament) in SDS sample buffer without a reducing agent were resolved by 10% SDS-PAGE. All SDS-PAGE gels were analyzed in duplicate with one set of samples supplemented with 10% 2-mercaptoethanol to confirm that any S-glutathiolation signal detected was reversible. After electrophoresis samples were transferred to PVDF membranes using a Bio-Rad semi-dry blotter.

In studies where myocytes were treated with unlabeled GSSG or diamide, S-glutathiolated proteins were identified using a mouse monoclonal antibody to S-glutathiolated proteins (anti-GSH, ViroGen.
Correlation of uninterpreted spectra to entries in Swiss-Prot/TrEMBL interfaced to a Micromass CapLC capillary chromatograph. Samples acceleration time of flight spectrometer (Micromass, Manchester, UK) spectra were recorded using a Q-TOF hybrid quadrupole/orthogonal 0.1% formic acid for mass spectrometry. Tandem electrospray mass mega, Madison, WI). Extracts were lyophilized and redissolved in ized by treatment with iodoacetamide. Gel pieces were dehydrated tonitrile, and cysteine residues were reduced with DTT and derivat-
ized by 10-fold in 50 mM Tris-HCl, pH 7.5 + 0.1% Triton X-100 to reduce the SDS concentration to 0.1%, thus avoiding denaturing of the antibody used later in the affinity purification step. The myofi-
ament fraction was also diluted 10-fold as above and then passed through a 0.2-μm syringe filter to remove any insoluble matter. Biotin-
GSSG-treated fractions were rotated overnight at 4 °C with strepta-
vidin-agarose beads, whereas GSSG-treated fractions were incu-
ubated with the anti-GSH-conjugated agarose beads. The beads were then collected in 2-ml columns containing a porous disc and washed with 50 ml of Tris-HCl, pH 7.5 + 0.1% SDS. Finally, proteins bound via disulfide formation were eluted using wash buffer supplemented with DTT (20 mM). SDS sample buffer containing iodoacetamide (final concentration, 100 mM) was added to the eluate, and proteins were separated by a non-reducing 4–15% gradient SDS-PAGE gel (Bio-
Rad) and stained with Simply Blue Coomassie stain (Invitrogen). Visible protein bands were then excised for LC-MS/MS analysis.

Identification of Unknown Proteins Using LC-MS/MS—In-gel di-
gestion of trypsin was performed according to published methods (11–13) modified for use with a robotic digestion system (Investigator ProGest, Genomic Solutions, Huntington, UK) (14). Briefly the Coomassie stain was removed from the excised gel pieces by sequential washing with 50 mM ammonium hydrogen carbonate buffer and acetonitrile, and cysteine residues were reduced with DTT and derivat-
ized by treatment with iodoacetamide. Gel pieces were dehydrated with acetonitrile and dried prior to addition of modified trypsin (Pro-
mega, Madison, WI). Extracts were lyophilized and redisolved in 0.1% formic acid for mass spectrometry. Tandem electrospray mass spectra were recorded using a Q-TOF hybrid quadrupole/orthogonal acceleration time of flight spectrometer (Micromass, Manchester, UK) interfaced to a Micromass CapLC capillary chromatograph. Samples were dissolved in 0.1% aqueous formic acid, injected onto a Pepmap C18 column (300 μm × 0.5 cm; LC Packings, Amsterdam, Netherlands), and eluted into the mass spectrometer with an acetonitrile, 0.1% formic acid gradient (5–70% acetonitrile over 20 min).

The capillary voltage was set to 3,500 V, and data-dependent MS/MS acquisitions were performed on precursors with charge states of 2, 3, or 4 over a survey mass range of 400–1300. Raw spectra were transformed onto a singly charged m/z axis using a maximum entropy method (MaxEnt3, Micromass). The Maxent parame-
ters were as follows: start mass = 700, peak width = auto, ensemble members = 1 (iterations = 20). Proteins were identified by correlation of uninterpreted spectra to entries in Swiss-Prot/TrEMBL using ProteinLynx Global Server (Version 1.1, Micromass). The database was created by merging the FASTA format files of Swiss-Prot, ProGest, Genomic Solutions, Huntington, UK) (14). Briefly the Coomassie stain was removed from the excised gel pieces by sequential washing with 50 mM ammonium hydrogen carbonate buffer and acetonitrile, and cysteine residues were reduced with DTT and derivat-
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RESULTS

Detection of S-Glutathiolated Proteins Using an Anti-GSH Antibody in Cardiac Myocytes—To make valid comparisons of the amount of S-glutathiolation present in a sample after various treatments, equal amounts of total protein were loaded in lanes where direct comparisons of signal intensities were made.

Fig. 2 is a non-reducing Western blot probed with an anti-GSH antibody and shows protein S-glutathiolation in subcellular fractions of myocytes under control conditions or after treatment with GSSG or diamide. Increased protein S-glutathiolation was particularly prominent in the myofilament fraction and also was present to a lesser extent in the membrane fraction following either of these treatments. There was little evidence of protein glutathiolation in the cytosol following GSSG treatment, whereas diamide did induce these modifications to some extent. The S-glutathiolation detected was abolished following treatment with the reducing agent 2-mercaptoethanol (not shown), confirming that the signal involved disulfide bonds, which helps to verify the specificity of the antibody. Diamide was used as a thiol-selective oxidant, which is known to promote protein S-glutathiolation. This diazene carbonyl derivative promotes radical-independent S-glutathiolation by undergoing an addition reaction with thiols on proteins or GSH itself (15). These adducts are then reduced by another GSH molecule, directly causing protein S-glutathiolation or the formation of GSSG. This GSSG can then undergo thiol-disulfide exchange reactions to further promote protein S-glutathiolation.

![Western blot images showing S-glutathiolation in various tissue samples.](https://www.mcponline.org)

**Fig. 3.** Protein S-glutathiolation induced by biotin-GSSG was detected in various tissues on non-reducing Western blots probed with streptavidin-HRP. Signals were abolished by the addition of 2-mercaptoethanol to each of the samples. Panel a, heart and lung; panel b, kidney and brain; panel c, skeletal muscle and liver.
Detection of S-Glutathiolated Proteins in Tissues Using Biotin-GSSG—Tissue homogenates from heart, lung, kidney, brain, skeletal muscle, and liver were treated with biotin-GSSG for 10 min. Fig. 3 shows non-reducing Western blots probed with streptavidin-HRP to detect proteins undergoing S-glutathiolation following this treatment. **Panel a** shows heart and lung samples in the absence (non-reducing) or presence (reducing) of 2-mercaptoethanol. Clearly there are a number of proteins in both heart and to a lesser extent lung that become S-glutathiolated in the presence of biotin-GSSG, and this labeling was fully reversed by a reducing agent. Similar results are seen in panels b and c for kidney, brain, skeletal muscle, and liver with individual, idiosyncratic S-glutathiolation patterns for each tissue. This demonstrates the utility of biotin-GSSG in the detection of S-glutathiolation targets in a range of tissues.

Detection of S-Glutathiolated Proteins in Cardiac Myocytes—Fig. 4 shows Western blots probed with streptavidin-HRP to detect proteins that were S-glutathiolated following biotin-GSSG treatment of cardiac myocytes. **Panel a** shows dose-dependent S-glutathiolation following treatment with a range of concentrations of biotin-GSSG (0–10 mM). On the basis of these results and to attempt to replicate cellular GSH:GSSG ratios during oxidant stress, subsequent studies were carried out using biotin-GSSG at 5 mM. **Panel b** shows the subcellular distribution of biotinylated proteins following biotin-GSSG treatment. S-Glutathiolated proteins were observed in all three subcellular fractions prepared. The signals generated were abolished following treatment with a reducing agent except for a prominent band at 75 kDa that likely represents an endogenously biotinylated protein. It is very clear that significantly more S-glutathiolated proteins are observed in all three subcellular fractions analyzed following biotin-GSSG treatment (Fig. 4, panel b) and analysis with streptavidin-HRP compared with equivalent experiments using unlabeled GSSG and detection with the monoclonal antibody (Fig. 2). The detection of significantly more S-glutathiolated proteins using the method involving biotin-GSSG is not simply
explained by longer exposure times or differences in the sensitivity achieved by the two HRP conjugates used in their detection. On a practical level this is evidenced by relatively low signal and quantity of bands using the antibody-based approach despite trials with increased antibody concentration, incubation, and exposure times. In addition to more intense signals, the biotin-GSSG method clearly detects significantly greater numbers of unique S-glutathiolated proteins as evidenced by the crowded banding pattern.

Fig. 5 shows the extent of protein S-glutathiolation by biotin-GSSG treatment following a pretreatment with unlabeled GSSG in heart homogenate. It is clear that GSSG treatment prior to biotin-GSSG reduces the amount of proteins that become biotinylated. This indicates that biotin-GSSG induces S-glutathiolation of the same protein as unlabeled GSSG.

Purification and Identification of S-Glutathiolated Proteins—Fig. 6 shows a Coomassie-stained SDS-PAGE gel of S-glutathiolated proteins purified by immunoprecipitation or by streptavidin-agarose affinity chromatography. Clearly the efficiency of the methods utilizing biotin-GSSG and streptavidin-agarose is much greater than that using the monoclonal antibody to S-glutathiolated proteins.

Proteins purified by immunoprecipitation, probably reflecting their low abundance. However, many of the proteins purified using the biotin-GSSG and streptavidin-agarose were identified; they are shown in Table I along with the peptide sequences used to identify them. Proteins identified include metabolic enzymes such as glyceraldehyde-3-phosphate dehydrogenase (G3P_RAT, bands 16 and 30), creatine kinase (KCRS_RAT, bands 15, 19, 24, and 29) triose-phosphate isomerase (TPIS_RAT, band 17), and trifunctional enzyme α subunit (ECHA_RAT, band 26). A number of myofilament proteins were also identified including myosin-binding protein C (MYPC3_MOUSE, band 23) and a protein similar to myosin-binding protein C (gi/H20841626645718, band 10), desmin (DESM_RAT, band 28), myosin heavy chain (MYH6_RAT, band 21), similar to M-protein (gi/H20841626662968, band 22), and α-actinin (gi/H20841626663741, band 25). In addition a protein similar to the human protein Met-induced mitochondrial protein (AAQ88168, band 33) was also identified. As shown in Table I some assignments are based on data derived from a single peptide. Where this is the case we have included each of the mass spectra used to make the identification as supplemental data. Several prominent protein bands remain unidentified, and because of the large number of proteins that can be modified in this way it is difficult to speculate about their identity.

Confocal Imaging of S-Glutathiolated Proteins—Representative confocal images of S-glutathiolated proteins in isolated rat ventricular myocytes are shown in Fig. 7. The images show that cells treated with biotin-GSSG display widespread protein S-glutathiolation as evidenced by the intense signals generated by ExtrAvidin-FITC staining that are not present in
S-Butyrylated proteins following biotin-GSSG treatment

Proteins purified following biotin-GSSG treatment were identified using LC-MS/MS. The peptide sequences and MASCOT score used to identify the proteins are also shown. The residues shown in parentheses are amino acid residues that we did not actually determine but can be deduced from database sequences. We have included them to illustrate that all the peptides used to make identifications are bona fide tryptic fragments with either arginine (R) or lysine (K) residues at the carboxyl terminus.

| Band | ID                        | Combined MASCOT score | Sequence                                        | MASCOT score | m/z precursor ion | Charge |
|------|---------------------------|-----------------------|-------------------------------------------------|--------------|------------------|--------|
| 10   | gi|62645718 similar to myosin-binding protein C (Rattus norvegicus) | 32          | (R)QAPPSEYER(I)                               | 32           | 538.77           | 2      |
| 15   | KCRS_RAT creatine kinase   | 221                   | (R)GLKEVER(L)                                  | 17           | 415.75           | 2      |
|      |                            |                       | (K)FEELTR(L)                                   | 32           | 453.77           | 2      |
|      |                            |                       | (K)LSVEALNSLTGFK(G)                            | 56           | 754.41           | 2      |
|      |                            |                       | (R)LGYLTPSNLGTGLR(A)                           | 40           | 867.99           | 2      |
|      |                            |                       | (R)LGGSEVOQQLVVDGVK(L)                         | 96           | 893.48           | 2      |
|      |                            |                       | (R)EVENVAILEKGDLAGR(Y)                         | 53           | 685.71           | 3      |
|      |                            |                       | (R)GTGGVDTAAADVYDISNIDR(I)                     | 106          | 1055.02          | 2      |
| 16   | G3P_RAT glyceraldehyde-3-phosphate dehydrogenase | 143 | (K)KVNGNGFR(I)                               | 44           | 403.23           | 2      |
|      |                            |                       | (−)KVNGNGFR(I)                                 | 29           | 516.81           | 2      |
|      |                            |                       | (R)GAANQLIAPTGSTGAAK(A)                        | 34           | 685.39           | 2      |
|      |                            |                       | (K)LVINGKPIITFQER(D)                           | 36           | 543.33           | 3      |
| 17   | TPIS_RAT triose-phosphate isomerase | 67 | (K)VFVETQTK(A)                               | 29           | 425.74           | 2      |
|      |                            |                       | (K)FFVGNNWK(M)                                 | 11           | 477.75           | 2      |
|      |                            |                       | (K)CNVSGEVAOCR(T)                              | 27           | 690.82           | 2      |
| 19   | KCRS_RAT creatine kinase   | 53                    | (R)EVENVAILEKGDLAGR(Y)                         | 53           | 685.71           | 3      |
| 21   | MYH6_RAT myosin heavy chain| 326                   | (R)INATLETQ(K)                                 | 27           | 445.26           | 2      |
|      |                            |                       | (K)LOFENGAR(L)                                 | 32           | 565.80           | 2      |
|      |                            |                       | (R)TKYETDAIQR(T)                               | 20           | 612.83           | 2      |
|      |                            |                       | (K)LASADIYELK(8)                               | 75           | 733.39           | 2      |
|      |                            |                       | (R)IEELLEELAE(R)                               | 37           | 744.88           | 2      |
|      |                            |                       | (R)ILNPAAPEGQFIDS(R)                           | 43           | 870.98           | 2      |
|      |                            |                       | (K)GTLEDIOQIANPALEAFGNAK(T)                    | 98           | 1100.56          | 2      |
| 22   | gi|62662968 similar to M-protein (R. norvegicus) | 166 | (R)LTVEGYTEGLSYFK(I)                           | 71           | 899.98           | 2      |
|      |                            |                       | (R)FLSGLAQLENVQAR(T)                           | 97           | 930.51           | 2      |
| 23   | MYPC3_MOUSE myosin-binding protein C | 304 | (K)LCETEGR(V)                                | 28           | 489.25           | 2      |
|      |                            |                       | (K)QQVLTLELR(K)                                | 56           | 514.82           | 2      |
|      |                            |                       | (R)NTPDITLFI(R)                                | 55           | 638.87           | 2      |
|      |                            |                       | (R)TSGGALAEVLQOEK(K)                           | 117          | 772.42           | 2      |
|      |                            |                       | (K)LEAPEAEDWVEILR(Q)                           | 51           | 835.45           | 2      |
|      |                            |                       | (K)VTPAPPEK(A)                                 | 21           | 484.27           | 2      |
| 24   | KCRS_RAT creatine kinase   | 157                   | (K)PPPLPOFGR(K)                                | 17           | 554.33           | 2      |
|      |                            |                       | (R)EVENVAILEKGDLAGR(Y)                         | 43           | 685.71           | 3      |
|      |                            |                       | (R)GTGGVDTAAADVYDISNIDR(I)                     | 99           | 1055.00          | 2      |
| 25   | gi|62663741 actin α-2 (R. norvegicus) | 123 | (K)IQVQSYI(R)                                | 24           | 483.29           | 2      |
|      |                            |                       | (R)LASELLEWIR(R)                               | 50           | 615.36           | 2      |
|      |                            |                       | (R)VGEWELLLTTIAR(R)                            | 28           | 686.41           | 2      |
|      |                            |                       | (R)ILADKPYLAEELR(R)                            | 28           | 577.68           | 3      |
| 26   | ECHA_RAT trifunctional enzyme α subunit | 64 | (K)TGLEQGNDAGYLAESKEFGELALTKE(E) | 64 | 881.11 | 3 |
| 28   | DESM_RAT desmin            | 42                    | (R)TFGGAPFSLGSPLSSPVFPR(A)                     | 45           | 1039.57          | 2      |
| 29   | KCRS_RAT creatine kinase   | 263                   | (K)VPPLPOFGR(K)                                | 18           | 554.33           | 2      |
|      |                            |                       | (R)EVENVAILEKGDLAGR(Y)                         | 41           | 743.42           | 2      |
|      |                            |                       | (R)LGYILTPSNLGTGLR(A)                          | 40           | 867.97           | 2      |
|      |                            |                       | (R)EVENVAILEKGDLAGR(Y)                         | 71           | 685.72           | 3      |
| 30   | G3P_RAT glyceraldehyde-3-phosphate dehydrogenase | 34 | (R)GAANQLIAPTGSTGAAK(A)                      | 34           | 685.40           | 2      |
| 33   | AAQ88168 Met-induced mitochondrial protein long (Homo sapiens) | 26 | (R)GLFTGLTPR(L) | 26 | 481.28 | 2 |
Study of Protein S-Glutathiolation Using Biotin-GSSG

Methods for Studying Protein S-Glutathiolation—A number of methods are available for the study of protein S-glutathiolation in cells, tissues, and in vitro models of oxidative stress that were recently reviewed in detail (17). Metabolic labeling of the GSH pool with radioactive $^{35}$S has been extensively utilized over several decades and has also been coupled to modern proteomic technology to allow identification of proteins that are S-glutathiolated during oxidative stress (18). S-Glutathiolation alters the charge of a protein, which facilitates detection of oxidized proteins using Western immunoblotting of samples separated by IEF gel electrophoresis (19). S-Glutathiolation induces IEF gel shifts that are reversed by treatment with reducing agents such as DTT. However, the application of this method is limited because it can only be used to study known targets of S-glutathiolation, the protein also has to be amenable to separation by IEF, and an antibody to it has to be available.

Previously we and others have used N-biotinyl analogues of reduced cysteine, GSH, or GSH ethyl ester in the study of protein S-thiolation (20–25). Using these compounds we studied protein S-thiolation in isolated organ preparations during ischemia and reperfusion. The oxidative stress, which accompanies ischemia and reperfusion, provided the driving force for protein S-thiolation with only a low relative level of basal S-thiolation in control preparations. One problem with the use of these compounds in in vitro or cell culture systems is that it is difficult to adequately replicate a pathophysiologically relevant oxidative stress. Many diseases with an oxidative component are modeled simply by treatment of isolated organs or cell cultures with metabolic inhibitors, chemical oxidants, or free radical-generating systems. Consequently the (patho)physiological significance of changes in such systems is questionable, and it is difficult to be sure of the mechanism by which the oxidant manifests a measured functional effect. This is because the introduced oxidant will likely react with many biomolecules, not solely with protein thiols to induce S-glutathiolation.

Antibody-based approaches for the detection and study of protein S-glutathiolation are particularly attractive because of the ability of many laboratories to undertake analysis involving Western immunoblotting, immunoprecipitation, and immunolocalization. Pan-specific antibodies that detect S-glutathiolated proteins using these methodologies have been reported (26–29). However, there may be some disadvantages to the use of these antibodies as considered below.

The Utility of Biotin-GSSG in the Study of Protein S-Glutathiolation—In these studies we overcame some of these limitations of the use of N-biotinyl-labeled reduced thiols by using biotin-GSSG instead. The treatment of cells with this compound replicates one specific consequence of oxidative stress, namely an increase in GSSG. Increased biotin-GSSG, as with unlabeled GSSG, promotes protein S-glutathiolation, and the biotin tag offers the advantage that it allows detection of these events as well as purification and identification.
of the proteins that become modified using avidin-based procedures.

Biotin-GSSG treatment of isolated adult, rat myocytes induced significant concentration-dependent protein S-glutathiolation, demonstrating the applicability of this method to cultured cell models. In addition by directly administrating biotin-GSSG in vitro to homogenates of the major organ systems, it was clear that extensive S-glutathiolation of proteins occurred in most tissue and that there was characteristic oxidation patterns for each. However, it was notable that significantly less S-glutathiolation occurred in lung tissue. This may reflect fewer targets or more probably target cysteines that are already oxidized under basal conditions and therefore unable to react with biotin-GSSG. In line with this, pretreatment of heart tissue with unlabeled GSSG immediately prior to addition of biotin-GSSG treatment decreased S-glutathiolation detected using streptavidin-HRP. This highlights an important point, that biotin-GSSG and GSSG broadly S-glutathiolate the same targets. This is of course a valuable feature of the biotinylated analogue, and the lack of interference by the biotin tag may be because it is adequately distant from the disulfide bond that undergoes exchange with protein thiols. In fact, the biotin tag is linked via an extended spacer arm to the amino terminus of the glutamate residue of glutathione, which increases the distance between the disulfide and the N-biotinyl moiety. It is possible that the biotin moiety will prevent labeling of some proteins due to steric hindrance, but our observations indicate this is not a major issue.

Comparison of Biotin-GSSG with Antibody-based Analysis—Several antibodies are available that are immunoreactive with glutathione and have established utility in ELISA for the quantitation of free glutathione or the immunolocalization of cellular free GSH and of S-glutathiolated proteins (26, 27, 29). To our knowledge there is only one commercially available antibody (product 101-A from ViroGen Corp.) that can be used for immunodetection of S-glutathiolated proteins on Western blots and in fixed cells or tissue sections as well as for immunoprecipitation. The full strategy for the generation of this antibody is unavailable as it is proprietary information. However, it is known that it involved immunizing mice with a keyhole limpet hemocyanin-GSH protein antigen in which the GSH was attached via its thiol group. This antibody, which was utilized in these studies, has been used in several recent studies to detect S-glutathiolation of actin (28), tubulin (30), and γ-crystallin (31), proteins that are notable by their high cellular abundance.

A comparison of the extent and intensity of protein S-glutathiolation detected using the two analytical methods indicates the biotin-GSSG methodology offers important advantages over the antibody-based approach. The biotin-GSSG approach allows the detection of significantly more S-glutathiolation substrates with significantly stronger signals observed on Western blots. Attempts to enhance the signal intensity of immunodetection on Western blots (by increasing the amount of antibody used or probing for longer) resulted in high background and the generation of bands that were not sensitive to DTT treatment that probably do not represent S-glutathiolated proteins.

Another issue regarding the use of antibodies is that their affinity for S-glutathiolation sites will differ because the exact makeup of their surrounding epitope will be variable. A variation in the affinity is to be expected with a pan-specific antibody as the modification it detects can occur at structurally varied locations. The biotin-GSSG methodology does not experience these problems as it relies on the extremely high affinity of biotin with the avidin and its derivatives and is unlikely to be substantially modulated by its molecular environment.

Confocal fluorescence imaging of cells corroborated the observations from the immunoblotting studies. Biotin-GSSG-treated cells, in which protein S-glutathiolation was detected using ExtrAvidin-FITC, displayed widespread and intense fluorescence. However, following GSSG treatment and classical immunostaining analysis, protein S-glutathiolation in cells was limited predominantly to the sarcolemma. This probably reflects the inability of GSSG, as with GSH, to cross membranes due to its charge. This is a point that is supported by the fact that diamide, which is membrane-permeable, induced more cytosolic protein S-glutathiolation than GSSG treatment. Clearly the addition of the biotin moiety to the amino groups of GSSG overcomes this cell permeability problem as biotin-GSSG enters the cell with great efficiency and promotes widespread efficient S-glutathiolation. These observations are further supported by the subcellular fractionation data from biotin-GSSG-treated cells in which proteins from all compartments analyzed, including the cytosol, were S-glutathiolated. This is in contrast to the pattern in cells treated with unlabeled GSSG where most of the signal is found in the membrane or Triton-insoluble fraction, consistent with extracellular labeling, as observed by immunofluorescence localization. The advantage of the biotin-GSSG method over those utilizing antibodies was also apparent from our purification studies. Significant numbers of S-glutathiolated proteins, in adequate abundance to allow good staining with colloidal Coomassie Blue, could be purified using streptavidin affinity chromatography of the cytosol, membrane, and myofilament fractions. Consequently this allowed a significant number of these purified proteins to be identified using LC-MS/MS analysis of tryptic digests and database searching. However, no identifications could be made following purification involving immunoprecipitation. This failure was because only very small, inadequate amounts of proteins were purified using this approach despite the use of a large amount of antibody-agarose conjugate.

It is clear that biotin-GSSG offers a useful tool for study of protein S-glutathiolation with some significant advantages over other analytical approaches, including those utilizing an-

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2 ViroGen Corp., personal communication.
tibodies. However, the use of biotin-GSSG is limited to the study of systems devoid of an endogenous oxidative stress as it is the disulfide itself that provides the oxidative driving force, resulting in protein S-glutathiolation. Consequently biotin-GSSG cannot be used to assess protein S-glutathiolation in cell or tissue samples with an intrinsic oxidative stress. For example, the screening of diseased tissues samples for glutathiolation could not be achieved using biotin-GSSG, whereas an antibody-based approach might prove fruitful. However, pan-specific S-glutathiolation antibodies are limited, as discussed above, by their inability to detect all classes of S-glutathiolation substrates. The addition of biotin-GSSG allows a defined component of oxidative stress to be investigated under carefully controlled conditions in the relative absence of other interfering redox reactions. Therefore biotin-GSSG can be used in models where an altered GSH:GSSG ratio has been shown previously to be important. In such studies, controlled or titrated amounts can be added to cells or tissue that directly compare with those measured in the disease states or other circumstances where the GSH:GSSG ratio is altered.

**S-Glutathiolated Proteins Identified in These Studies—**

Some of the proteins identified were previously shown to be S-glutathiolate substrates in our earlier studies (20, 22, 23), including GAPDH, creatine kinase, myosin heavy chain, triose-phosphate isomerase, and trifunctional enzyme α subunit. However, myosin-binding protein C, Met-induced mitochondrial protein, α-actin 2, desmin, and myomesin 2 are previously unknown targets of cysteine-targeted oxidation. Many of the proteins identified are metabolic enzymes or myofilament components, two groups of proteins present in high abundance in cardiac myocytes. It is likely that there are significant numbers of low abundance proteins that are targets of S-glutathiolation that could be identified in future studies encompassing modifications of the methods used. Such methodological alterations might increase the chromatographic resolution of the purified S-glutathiolated proteins and also enhance the sensitivity of their detection prior to LC-MS/MS analysis.

In conclusion, we demonstrated the utility of biotin-GSSG in the detection, purification, and visualization of proteins that undergo S-glutathiolation. The novel experimental approaches we developed may find widespread utility in the proteomics of glutathionylated proteins in oxidatively stressed human T lymphocytes.

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