Detection, Quantitation, Purification, and Identification of Cardiac Proteins S-Thiolated during Ischemia and Reperfusion*

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Philip Eaton‡, Helen L. Byers§, Nicola Leeds§, Malcolm A. Ward§, and Michael J. Shattock

From the Centre for Cardiovascular Biology and Medicine, The Rayne Institute, St Thomas’ Hospital, King’s College London, SE1 7EH and §Proteine Sciences plc, South Wing Laboratory, Institute of Psychiatry, King’s College London, Denmark Hill, SE5 8AF, United Kingdom

We have developed methods that allow detection, quantitation, purification, and identification of cardiac proteins S-thiolated during ischemia and reperfusion. Cysteine was biotinylated and loaded into isolated rat hearts. During oxidative stress, biotin-cysteine forms a disulfide bond with reactive protein cysteines, and these can be detected by probing Western blots with streptavidin-horseradish peroxidase. S-Thiolated proteins were purified using streptavidin-agarose. Thus, we demonstrated that reperfusion and diamide treatment increased S-thiolation of a number of cardiac proteins by 3- and 10-fold, respectively. Dithiothreitol treatment of homogenates fully abolished the signals detected. Fractionation studies indicated that the modified proteins are located within the cytosol, membrane, and myofibril/cytoskeletal compartments of the cardiac cells. This shows that biotin-cysteine gains rapid and efficient intracellular access and acts as a probe for reactive protein cysteines in all cellular locations. Using Western blotting of affinity-purified proteins we identified actin, glyceraldehyde-3-phosphate dehydrogenase, HSP27, protein-tyrosine phosphatase 1B, protein kinase Co, and the small G-protein ras as substrates for S-thiolation during reperfusion of the ischemic rat heart. MALDI-TOF mass fingerprint analysis of tryptic peptides independently confirmed actin and glyceraldehyde-3-phosphate dehydrogenase S-thiolation during reperfusion. This approach has also shown that triosephosphate isomerase, aconitate hydratase, M-protein, nucleoside diphosphate kinase B, and myoglobin are S-thiolated during post-ischemic reperfusion.

Oxidative damage contributes to the injury sustained by the heart during harmful periods of ischemia and reperfusion (1). Protein thiols have been implicated as targets of this oxidative attack (2), and it is likely that injury involves terminal or irreversible oxidation of these reactive groups. Less severe oxidative damage occurs during reperfusion after shorter sublethal periods of ischemia and reperfusion (ischemic preconditioning), and this initiates a stress-adaptive signaling response (3, 4).

Post-translational modification by S-thiolation can regulate protein function in much the same way as phosphorylation (5). Although protein S-thiolation is modulated by the cellular redox status, there are also specialized enzyme systems that catalyze the addition or removal of low molecular weight thiols (6). There are an increasing number of proteins that are known to be regulated by S-thiolation. Proteins regulated by cysteine oxidation include ion translocators (7), structural proteins (8), metabolic enzymes (9–11), DNA isomerases (12), and signaling proteins. Signal transduction proteins that are directly regulated by oxidative modification of cysteine residues include protein phosphatases (13), protein kinases (14, 15), G-proteins (16, 17), and membrane receptors (18).

The study of protein S-thiolation in vivo is technically challenging, and this has hindered progress in this area. Many studies of protein S-thiolation during oxidant stress have used cultured cells with a radiolabeled thiol pool in which the S-thiolated proteins are detected by autoradiography of non-reducing SDS-PAGE gels (19). The investigation of specific proteins that are known to be S-thiolated has been achieved by non-reducing isoelectric-focusing gel electrophoresis coupled with Western blotting (8). The basis of this approach is that protein S-thiolation changes the isoelectric point of proteins, which alters the isoelectric focusing banding pattern (an effect that is reversible by application of chemical reducing agents such as DTT). The methods we have developed overcome many of the difficulties in the investigation of protein S-thiolation.

Previously, we used biotinylated glutathione to detect and purify proteins that are S-glutathiolated during ischemia and reperfusion (20, 21). However, this probe for reactive cysteines was inefficient in crossing the plasma membrane, and primarily labeled membrane proteins. Here we report a refinement of this approach, which exploits biotinylated cysteine (biotin-cysteine) as a probe for proteins that are S-thiolated during oxidant stress. Biotin-cysteine rapidly crosses the plasma membrane and can be used to detect, quantify, and identify proteins susceptible to oxidation in all compartments of the cell.

Here we show that cytosolic and detergent-insoluble (myofilament and cytoskeletal) proteins are also major substrates for S-thiolation during the pro-oxidizing condition in myocardium. We have also been able to identify a number of the proteins that become S-thiolated in the myocardium following ischemia and reperfusion. S-Thiolation of proteins during ischemia and reperfusion is particularly significant (as this is a physiologically relevant oxidant stress), whereas many studies use chemical oxidants that cause a physiologically irrelevant redox change.

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† To whom correspondence should be addressed. Tel.: 020-7928-9292 (ext. 2749); Fax: 020-7922-8139; E-mail: philip.eaton@kcl.ac.uk.

† The abbreviations used are: DTT, dithiothreitol; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MALDI-TOF, matrix-assisted laser desorption/ionization-time of flight; HPLC, high pressure liquid chromatography.
pellet that represents cytoskeletal and myofilament proteins. Each of the three fractions was reconstituted in SDS buffer without a reducing agent. SDS-PAGE was carried out using the Bio-Rad mini protein II system. In some samples, to confirm that S-thiolated/biotinylated proteins were modified via disulfide formation, 20 mM DTT was added to the final SDS sample. After electrophoresis, samples were transferred to polyvinyldene difluoride using a semi-dry blotter (Amersham Biosciences, Inc.). S-Thiolated proteins were identified by virtue of their biotin tags using streptavidin-horseradish peroxidase (Amersham Biosciences, Inc.) and the enhanced chemiluminescence reagent (Amersham Biosciences, Inc.). Western blots were digitized using a flatbed scanner (HP Scanjet 11C). The digitized image was then quantitatively analyzed for total protein S-thiolation in each lane using the NIH-Image software.

**Purification of S-Thiolated Proteins—** S-Thiolated proteins were affinity-purified using streptavidin-agarose. Hearts were homogenized (10 ml of buffer per g of cardiac tissue) on ice in 100 mM Tris-HCl, 5 mM EGTA, 5 mM EDTA, benzamidine (10 μg/ml), leupeptin (100 ng/ml), and aprobin (100 ng/ml)/pH 7.0 using a polytron tissue grinder. The cytosolic and membrane fractions were used directly for the purification of the oxidized protein. The cytoskeletal and myofilament pellet proteins were solubilized by resuspension in homogenization buffer containing 1% SDS. Each fraction was rotated at 4°C overnight. The agarose pellet was then extensively washed with phosphate buffered saline and 1% Triton X-100. S-Thiolated proteins were released from the streptavidin-agarose by treatment with 20 mM DTT in phosphate buffered saline.

**Identification of Unknown S-Thiolated Proteins by Immunoblotting—** Affinity-purified proteins were reconstituted in SDS sample buffer and resolved by SDS-PAGE. After transfer to polyvinylidene difluoride membrane we tested for the presence of candidate proteins. The proteins were selected on the basis that there is previous evidence in the literature suggesting that they are targets for thiol modification. The presence of these proteins was tested using standard immunoblotting methods. The antibodies used included actin (Sigma), GAPDH (Biogenesis, UK), HSP27 (Stressgen Biotechnologies Ltd., distributed by Biotype Ltd., York, UK), protein-tyrosine phosphatase 1B (Santa Cruz Biotechnology Inc.), protein kinase Co (Transduction Laboratories, Franklin Lakes, NJ), and Ras protein (Transduction Laboratories).

**Identification of Unknown S-Thiolated Proteins by Mass Spectrometry—** Tryptic digests were analyzed by matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry using a Voyager DE-Pro instrument operated in reflectron mode. All spectra were internally calibrated using a lock mass routine based on the monoisotopic molecular ion relating to a tryptic autolysis product present at m/z 2183.569. A list of monoisotopic peptide masses was obtained for each sample. These were submitted to the Mascot data base searching algorithm. Each protein was identified by matching multiple peptides to the corresponding entry within the SwissProt data base. A mass accuracy of 30 ppm was specified.

**HPLC Analysis of Affinity-purified Proteins—** A Shimadzu HPLC system, equipped with diode array detection and automated fraction collection was utilized. Separation was carried out using a Hewlett Packard Zorbax 300SB C8 reverse phase column, an optimized trifluoroacetic acid/acetonitrile gradient and detection at 280 nm.

**Statistics—** Results are presented as mean ± S.E. Differences between groups were assessed using analysis of variance followed by a Bonferroni t test. Differences were considered significant at the 95% confidence level.

**RESULTS**

**Protein S-Thiolation during Myocardial Oxidant Stress—** Fig. 2A shows that there was a low, basal level of protein S-thiolation in the aerobically perfused isolated rat heart and that ischemia and reperfusion or treatment with the thiol-selective oxidant diamide increased the amount of protein oxidation. Quantitative analysis of total protein S-thiolation (Fig. 2B) shows that the physiologically relevant oxidative burden of ischemia and reperfusion significantly (p < 0.05) increased the modified protein (3-fold). The thiol-selective oxidant diamide was particularly effective at inducing protein S-thiolation; significantly (p < 0.05) increasing it 10 fold above control and DTT treatment of these samples abolished S-thiolation.

**Subcellular Localization of S-Thiolated Proteins—** Fig. 3 shows that proteins from cytosolic, membrane, and cytoskeletal/myofilament fractions were all S-thiolated during ische-
mia and reperfusion. The largest overall amount of S-thiolation occurred in the cytosolic and cytoskeletal/myofilament fractions.

**Purification of S-Thiolated Proteins**—Fig. 4A shows a Coomassie-stained gel of S-thiolated proteins that were affinity-purified using streptavidin-agarose from a cardiac homogenate treated with SDS, which will solubilize all of the protein components present. Clearly there are S-thiolated proteins over the entire molecular weight range of this 10% acrylamide gel, and this represents the S-thiolation of a great many proteins (possibly hundreds) during cardiac reperfusion. HPLC analysis of S-thiolated proteins purified from the cytosol (Fig. 4B) shows that there are about twenty dominant S-thiolation substrates in this fraction.

**Identification of S-Thiolated Proteins Using Immunoblotting**—Fig. 5 shows a series of Western immunoblots in which we have used a panel of antibodies to probe the affinity-purified S-thiolated proteins. The antibodies used were selected because they probe for proteins that have been described previously to be susceptible to cysteine-targeted oxidative modifications. This study shows that proteins that are S-thiolated during cardiac reperfusion include actin, GAPDH, HSP27, protein-tyrosine phosphatase 1B, protein kinase C, and the small G-protein ras. At the present time, the majority of the proteins oxidized at cysteine residues during reperfusion remain unknown. Further analysis of the affinity-purified sample may identify proteins that are not yet known to be regulated by S-thiolation.

**Identification of S-Thiolated Proteins Using MALDI-TOF Mass Spectrometry**—Delayed extraction MALDI mass fingerprint analysis of tryptic peptides identified four of the purified S-thiolated proteins. Fig. 6 shows a representative peptide mass fingerprint obtained after in-gel digestion of a 25-kDa S-thiolated protein shown in Fig. 4A. 12 peptides were found to match those expected for triosephosphate isomerase, which represents 53% sequence coverage. Other proteins identified in this way included GAPDH, actin, aconitate hydratase, M-protein, nucleoside diphosphate kinase B, and myoglobin. It is noteworthy that the immunoblot analysis, which also identified actin and GAPDH as S-thiolation substrates, was carried out prior to the MALDI-TOF analysis.

**DISCUSSION**

**The Utility of Biotin-Cysteine for Detection, Localization, Purification, and Identification of S-Thiolated Proteins**—In this study we have shown the practical application of biotin-cysteine in the investigation of protein S-thiolation. These methods will find application in isolated organs, tissues, cell cul-
tures, or homogenates where redox-induced protein S-thiolation is of interest. The presence of a biotin tag on proteins that become S-thiolated allowed a range of investigative procedures to be carried out, which exploit the high affinity of biotin for avidin derivatives. Thus, S-thiolated proteins were detected on non-reducing Western blots using streptavidin-horseradish peroxidase and chemiluminescence and were quantified via digitization and pixel intensity analysis. S-Thiolated proteins were purified using a streptavidin affinity matrix and subsequently eluted using DTT. These proteins were then identified using Western blotting and antibodies to candidate proteins that are known to have reactive cysteines or MALDI-TOF mass spectral analysis of tryptic peptides involving protein data base searching. N-terminal sequencing and public data base searching of the purified proteins are also suitable methods for identifying each protein. The subcellular fractionation studies showed that membrane, cytoskeletal/myofilament, and cytosolic proteins become S-thiolated during reperfusion. The modification of cytosolic proteins at this time demonstrates that biotin-cysteine gains rapid intracellular access. Previously we used biotinylated glutathione to detect S-thiolated proteins, but we found that membrane proteins were the principal targets for modification and that few cytosolic proteins were labeled (20, 21). The biotin-GSH did not efficiently cross the plasma membrane, indicating the advantage of the present method utilizing biotin-cysteine. The use of avidin-coupled fluorescent dyes would also allow the localization of modified proteins using microscopy.

S-Thiolation during Cardiac Oxidant Stress and Functional Consequences—Clearly the pathophysiological oxidant stress associated with ischemia and reperfusion results in S-thiolation of a range of cardiac proteins. The amount of S-thiolation induced by the thiol-selective diamide was considerably greater than that induced by reperfusion, indicating that only a small proportion of proteins are S-thiolated during the ischemia/reperfusion protocol. Our findings agree with studies that have used myocytes or cell lines with 35S-labeled GSH to show that S-glutathiolation occurs following treatment with chemical oxidants, which simulates the oxidative component of ischemia and reperfusion (19). Protein S-thiolation during reperfusion is expected because free radicals are known to be produced at this time (22). These free radicals target thiol groups, rendering many proteins dysfunctional, and may contribute to myocardial stunning or reperfusion arrhythmias (1).

Protein cysteines can be modified by a range of electrophilic species with a spectrum of potential cysteine oxidation products, some of which are reversible. Reversible cysteine modifications including S-thiolation, can be regarded as a protective mechanism in which reactive protein thiols are protected from irreversible or terminal oxidation by the formation of a mixed disulfide with a low molecular weight thiol. The proteins that were modified by biotin-cysteine during reperfusion are likely to be representative of proteins with reactive cysteines that are particularly susceptible to oxidation in a number of different ways (not just S-thiolation). Thus, although we have detected S-thiolated proteins during early reperfusion, there may also be a variety of other oxidative cysteine modifications occurring at the same time. In the absence of biotin-cysteine, the modification may involve a range of species including glutathione, cysteine, homocysteine, nitrosoglutathione, glutathione sulfonamide, glutathione disulfide S-oxide, nitric oxide, hypochlorous acid, or other oxidizing species. This is illustrated by studies showing that cysteines within the G-protein Ha-Ras are susceptible to both S-thiolation and S-nitrosylation (17). Presumably, the oxidative cysteine end products that dominate during an oxidative insult will depend on many factors, including the nature and intensity of the oxidizing species and intracellular spatial distribution. Protein cysteines can also be progressively oxidized by oxygen to form sulfenic (SOH), sulfenic (SOOH) or sulfonyl (SO$_3$H). Sulfenic modifications are reversible and chemically reducible, and its formation is functionally critical in the activity and regulation of some proteins (23).

Whether or not a protein thiol undergoes reversible or irreversible oxidation will depend on whether it encounters a low molecular weight thiol or molecular oxygen as well as the extent of the oxidative insult. A greater oxidative burden may be expected after longer periods of ischemia because at this time low molecular weight thiol antioxidants are depleted, as are NADPH and ATP, which are required for the maintenance of many antioxidant defense systems (24). Thus, the proteins we have detected by virtue of an S-thiolation reaction are those that are likely to be oxidized (reversibly or irreversibly) during cardiac reperfusion. These proteins may represent the actual molecular targets of the oxidative damage that accompanies reperfusion and consequently may represent potential therapeutic targets. The formation of terminally oxidized protein thiols will have far reaching consequences because as these proteins will have to be replaced by de novo synthesis. Functionally critical reactive thiol groups are found in many key cardiac proteins including those that are important in ATP production (9), iron homeostasis (7), signal transduction (14, 16, 18), transcription (25), cellular redox control (9), and excitation-contraction coupling (26). The oxidation of these key proteins would have profound implications for cardiac function and viability during oxidative stress.

Mechanism of Protein S-Thiolation during Cardiac Reperfusion—There are multiple mechanisms by which protein S-thiolation may occur in the isolated rat heart during post-ischemic reperfusion. It is not clear whether a single mechanism is solely responsible for S-thiolation or if several mechanisms contribute. Metal-catalyzed oxidation or disulfide bond exchange are likely modes of protein S-thiolation in the heart. Biotin-cysteine is likely to form a range of low molecular weight mixed disulfides including self-dimerization and with endogenous cysteine, glutathione, or other cysteine-containing molecules. Once formed, these mixed disulfides will exchange with reduced protein thiols and cause S-thiolation.

Identification of Proteins S-Thiolated during Reperfusion—Commassie-stained SDS-PAGE analysis of S-thiolated proteins that have been affinity-purified demonstrates that a great many proteins become S-thiolated during oxidant stress. HPLC analysis of S-thiolated proteins from the cytosol shows that there are about twenty dominant proteins present. Western blot analysis of affinity-purified S-thiolated proteins with antibodies to candidate proteins has demonstrated that actin, GAPDH, HSP27, protein-tyrosine phosphatase 1B, protein kinase Ca, and the G-protein ras are S-thiolated during cardiac ischemia and reperfusion. MALDI-TOF mass spectrometry peptide mapping has also independently identified GAPDH and actin as target proteins for S-thiolation. In addition MALDI-TOF analysis has also identified triosephosphate isomerase and aconitate hydratase (aconitase) as oxidative targets. Clearly, the majority of the proteins that are S-thiolated during reperfusion remain unknown. Most of the S-thiolation...
substrates we have identified are proteins that have already been reported to have reactive cysteine residues susceptible to oxidation. Indeed there is evidence in cellular systems for the S-thiolation of actin, GAPDH, protein-tyrosine phosphatase 1B, protein kinase C, and the G-protein ras (8, 13, 15, 16, 27).

S-Thiolation of HSP27 and Actin—HSP27 S-thiolation has been previously observed in an in vitro system in which GSSG was added to purified protein. Cysteine 141 is the only thiol group within rat cardiac HSP27 and therefore must be the site of S-thiolation. These studies also showed that HSP27 formed disulfide-linked dimers and that these paired protein molecules assemble into the large multimeric complexes (28, 29). It has also been shown in a cell model that there is a direct relationship between the multimeric aggregate size of HSP27 and the concentration of GSH as well as evidence that there is interplay between the HSP27 and GSH that regulates the cellular levels of these molecules (30). The oligomeric state of HSP27 regulates its ability to act as a molecular chaperone and also controls its ability to inhibit the polymerization of actin, an intermediate filament protein that interestingly is also a known target of S-thiolation (8).

S-Thiolation of Metabolic Enzymes during Cardiac Reperfusion—Many metabolic enzymes are known to have reactive cysteine residues, the oxidation of which inactivates these proteins (31). Identification of GAPDH is consistent with previous reports (including our own, Ref. 32), that GAPDH is a target for oxidative stress (27, 33). It is likely that S-thiolation occurs at the reactive and catalytically essential cysteine 149 (34). Oxidation of this residue inactivates the enzyme and may be achieved by a range of oxidants including GSSG, GSNO, NO, HOCl, and GS(O)SG (33, 35–40). Triosephosphate isomerase is another glycolytic enzyme that we found to be S-thiolated during cardiac oxidative stress. It catalyzes the reversible interconversion of glyceraldehyde 3-phosphate (the same molecule metabolized by GAPDH) and dihydroxyacetone phosphate. In the disease hereditary hemochromatosis there is a systemic deficiency of triosephosphate isomerase, causing wide ranging cardiac pathologies (41). Inhibition of this enzyme is likely to contribute to the metabolic deficit that is known to occur during reperfusion (42). We also detected the oxidation of another isomerase during reperfusion, the enzyme aconitase. The iron sulfur-containing citric acid enzyme aconitase is responsible for the conversion of citrate to isocitrate. Aconitase activity is dependent on the redox state of its cubane [2Fe–4S] cluster as well as the surrounding cysteine residues. Oxidants such as peroxynitrite or hydrogen peroxide are known to reversibly inhibit this protein and may contribute to its loss of cardiac function during ischemia and reperfusion (11). Inhibition also contributes to other disease states such as neurodegeneration (43). Myocardial aconitase activity is lost during ischemia and reperfusion, but this can be prevented by antioxidant interventions with low molecular weight thiols. In this connection, antioxidant therapy with low molecular weight thiols is known to combat cardiac injury caused by ischemia and reperfusion.

Signal Transduction, Cardiac Stress Adaptation, and S-Thiolation—Protein kinase C isoforms are known to have cysteine-rich domains that contain target residues for S-thiolation or S-nitrosylation (32). The fact that GAPDH and aconitase are targets for S-thiolation suggests that it may be a common reaction which takes place during oxidative stress in many different cellular compartments. It is of interest that GAPDH is known to regulate the activity of many other enzymes, and that S-thiolation may affect the activity of these enzymes through modification of reactive cysteine residues.

Fig. 6. Delayed extraction MALDI peptide mass fingerprint obtained after in-gel digestion of a 25-kDa protein band. Public database searching identified the protein as triosephosphate isomerase with 12 matching peptides, representing 53% sequence coverage. We also identified triosephosphate isomerase, aconitate hydratase, M-protein, nucleoside diphosphate kinase B, and myoglobin as S-thiolated targets using this technique.
of cardioprotective stress adaptive pathways (3, 4). Similarly, the protection afforded by ischemic preconditioning is blocked by antioxidants and initiated by pro-oxidants (45, 46). Thus, it is possible that modification of cysteine residues in the heart during oxidative stress may be involved with the triggering of the preconditioning cardioprotection. In this connection, protein kinase C and protein phosphatases have both been implicated in the mechanism of ischemic preconditioning; thus, it is particularly interesting that we have detected the S-thiolation during ischemia and reperfusion of the heart (44).

Conclusions—Clearly biotin-cysteine is a useful tool in the study of protein S-thiolation. In these studies we have applied its application and utility in the setting of ischemia and reperfusion in the isolated rat heart. The methodologies we have developed are applicable to other intact organ models of oxidant stress as well as tissue culture and in vitro models.

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