Detection and Mapping of Widespread Intermolecular Protein Disulfide Formation during Cardiac Oxidative Stress Using Proteomics with Diagonal Electrophoresis*

Jonathan P. Brennan†, Robin Wait‡, Shajna Begum§, James R. Bell‡, Michael J. Dunn¶, and Philip Eaton†

From the †Department of Cardiology, Cardiovascular Division, King’s College London, The Rayne Institute, St. Thomas’ Hospital, London SE1 7EH, United Kingdom, the ‡Kennedy Institute of Rheumatology Division, Faculty of Medicine, Imperial College London, 1 Aspenlea Road, Hammersmith, London, W6 8LH, United Kingdom, and the §Department of Neuroscience, Institute of Psychiatry, King’s College London, London SE5 8AF, United Kingdom

Regulation of protein function by reversible cysteine-targeted oxidation can be achieved by multiple mechanisms, such as S-glutathiolation, S-nitrosylation, sulfinic acid, sulfonic acid, and sulfenyl amide formation, as well as intramolecular disulfide bonding of vicinal thiols. Another cysteine oxidation state with regulatory potential involves the formation of intermolecular protein disulfides. We utilized two-dimensional sequential non-reducing/reducing SDS-PAGE (diagonal electrophoresis) to investigate intermolecular protein disulfide formation in adult cardiac myocytes subjected to a series of interventions (hydrogen peroxide, S-nitroso-N-acetylpenicillamine, doxorubicin, simulated ischemia, or metabolic inhibition) that alter the redox status of the cell. More detailed experiments were undertaken with the thiol-specific oxidant diamide (5 mM), a concentration that induces a mild non-injurious oxidative stress. This increase in cellular oxidation potential caused global intermolecular protein disulfide formation in cytosolic, membrane, and myofilament/cytoskeletal compartments. A large number of proteins that undergo these associations were identified using liquid chromatography-mass spectrometry/mass spectrometry. These associations, which involve metabolic and antioxidant enzymes, structural proteins, signaling molecules, and molecular chaperones, were confirmed by assessing “shifts” on non-reducing immunoblots. The observation of widespread protein-protein disulfides indicates that these oxidative associations are likely to be fundamental in how cells respond to redox changes.

Oxidants have been implicated in the pathogenesis of most major diseases (1–4), including those of the heart (5). However, substantial contemporary evidence is emerging that shows that these species play important regulatory roles in the everyday function of cells and tissues (6–9). The roles of oxidants as signaling, or regulatory entities, that allow cell function to be coupled to its redox status is being increasingly established.

In terms of abundance, the most important redox couples within cells involve the equilibrium between reduced and oxidized forms of thiols. The status of the cellular reduction potential, which essentially is an index of the thiol redox status, varies during the life history of a cell. For example, the cellular reduction potential has characteristic set points depending on whether tissue is quiescent, proliferating, confluent, differentiating, or apoptotic (10, 11). Changes in cellular redox potential to a more oxidizing state can be triggered by a number of processes in healthy tissue, such as metabolism and signaling events. Under basal conditions neurohumoral substances not only activate classical signaling pathways, but also transactivate others that are dependent on the generation of endogenously derived oxidants such as superoxide, hydrogen peroxide, or nitric oxide (12–14). When the cellular oxidation potential is elevated, protein thiols within the cell become susceptible to modification. A number of reversible protein thiol oxidation states can be produced, including S-thiolated (15), S-nitrosylated (8), sulfinic acid (PSO) (16), sulfenic acid (PSO2H) (17), sulfenyl amide (18), and reactive oxidized lipid derivatives (19). Vicinal thiols, which form intramolecular protein disulfides, are also an established mode of cysteine-targeted oxidation that allows regulatory control (20).

Another form of protein-cysteine oxidation that is predicted to occur in cells under oxidizing conditions involves the formation of a disulfide bond between proteins. In this study we utilized two-dimensional sequential non-reducing/reducing SDS-PAGE (diagonal electrophoresis) (21), to separate proteins that form disulfides in myocardial cells under an oxidative burden. These proteins have then been identified using LC-MS/MS, and provide new insights as to how proteins and cells respond to changes in their redox environment.

MATERIALS AND METHODS

Cell Preparation—Calcium-tolerant ventricular myocytes were isolated from the hearts of male Wistar rats (250–300 g) using a standard collagenase digestion protocol as described previously (22). Animals received humane care in compliance with the Principles of Laboratory Animal Care formulated by the National Society for Medical Research and the Guide for the Care and Use of Laboratory Animals prepared by the National Institutes of Health (43). After isolation, myocytes were kept at room temperature in Tyrode buffer for 3 h after isolation before subsequent treatments.

Assessing Interventions That Induce Protein-Protein Disulfides—we screened a series of oxidative interventions for their ability to promote...
intermolecular protein disulfide bonds. Control or treated cells were pelleted at 500 × g for 0.5 min and resuspended in lysis buffer containing 50 mM Tris-HCl, pH 7.5, 5 mM EDTA, 2 mM NaF, 0.05% dithionit, 1% Triton X-100, and 100 mM maleimide and left on ice for 5 min. Soluble protein was collected by centrifugation (10,000 × g for 2 min) and reconstituted in SDS sample buffer without a reducing agent but with 100 mM maleimide. The sample was then analyzed by diagonal electrophoresis as described below. Interventions involved using this approach included treatment with diamide (5 mM for 10 min) or hydrogen peroxide (0.1, 1, or 10 mM for 10 min), S-nitroso-N-acetylenicillamine (SNAP, 0.01, 0.1, or 1 mM for 10 min), doxorubicin (0.001, 0.01, or 0.1 mM for 10 min), N-acetylcysteine (10 mM for 10 min), metabolic inhibition, or simulated ischemia. Metabolic inhibition was induced by treating cells in control buffer with dinitrophenol (0.01 mM) and with 25 mM ammonium hydrogen carbonate, 5% formic acid, and acetonitrile. The pooled extracts were lyophilized and resuspended in 0.1% formic acid for mass spectrometry.

Subcellular Fractionation Studies in Diamide-treated Myocytes—In separate experiments, cells were divided into two aliquots and treated for 10 min with diamide (final concentration 5 mM) or tyrode buffer alone as a control. Following treatment cells were pelleted at 500 × g for 0.5 min, the supernatant was removed, and cells were washed in lysis buffer. This was repeated, and the pellet was resuspended in lysis buffer containing 50 mM Tris-HCl, pH 7.5, 5 mM EDTA, 2 mM NaF, 0.05% dithionit, and 100 mM maleimide and left on ice for 5 min on ice with frequent vortexing. The samples were then spun at 10,000 × g for 2 min, and the supernatant was laid in 1% Triton X-100. The samples were spun again, and the supernatant was kept (membrane fraction). The Triton X-100 insoluble pellet (myofilament fraction) was washed and then resuspended in lysis buffer. Additional immunoblot studies with antibodies to marker proteins for each of these fractions (not shown) showed that this fractionation protocol was effective. Fractions were reconstituted in SDS sample buffer, free of reducing agents, containing 100 mM maleimide. Maleimide was present in all samples to block all free thiol groups and prevent thiol-disulfide exchange or thiol oxidation during preparation.

Sequential Two-dimensional Non-reducing/reducing SDS-PAGE—This method has been referred to as diagonal electrophoresis (21). Subcellular fractions (cytosolic, membrane, or myofilament in SDS sample buffer without a reducing agent) from control or diamide-treated preparations were resolved on 12% SDS-polyacrylamide gels (Bio-Rad mini protein III system). After non-reducing electrophoresis, the entire lane containing the resolved proteins was excised and reconstituted in SDS sample buffer containing 50 mM Tris-HCl, pH 7.5, 5 mM EDTA, 2 mM NaF, 0.05% dithionit, and 100 mM maleimide and left on ice for 5 min. Soluble protein was collected by centrifugation (10,000 × g for 2 min) and reconstituted in SDS sample buffer containing 50 mM Tris-HCl, pH 7.5, 5 mM EDTA, 2 mM NaF, 0.05% dithionit, and 100 mM maleimide and left on ice for 5 min on ice with frequent vortexing. The samples were then spun at 10,000 × g for 2 min, and the supernatant was laid in 1% Triton X-100. The samples were spun again, and the supernatant was kept (membrane fraction). The Triton X-100 insoluble pellet (myofilament fraction) was washed and then resuspended in lysis buffer. Additional immunoblot studies with antibodies to marker proteins for each of these fractions (not shown) showed that this fractionation protocol was effective. Fractions were reconstituted in SDS sample buffer, free of reducing agents, containing 100 mM maleimide. Maleimide was present in all samples to block all free thiol groups and prevent thiol-disulfide exchange or thiol oxidation during preparation.

Identification of Unknown Proteins Using LC-MS/MS—In-gel digestion with trypsin was performed according to published methods (23–25), modified for use with a robotic digestion system (Investigator ProGest, Genomic Solutions, Huntington, UK) (26). The Coomassie stain was removed from the excised gel pieces by sequential washing with 50 mM ammonium hydrogen carbonate buffer and acetonitrile. Cysteine residues were reduced with dithiothreitol and derivatized by treatment with iodoacetamide. After further washing with ammonium hydrogen carbonate buffer the gel pieces were dehydrated with acetonitrile and dried at 60 °C prior to addition of modified trypsin (Promega, 10 μl at 6.5 ng/μl in 25 mM ammonium hydrogen carbonate). Digestion proceeded for 8 h at 37 °C, and products were recovered by sequential extractions with 50 mM ammonium hydrogen carbonate, 5% formic acid, and acetonitrile. The pooled extracts were lyophilized and resuspended 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, and 6 μl was injected onto a Pepmap C18 column (300 μm × 0.5 cm; LC Packings, Amsterdam, Netherlands), and washed for 3 min with 0.1% aqueous formic acid (with the stream select valve diverting the column effluent to waste). The flow rate was then reduced to 1 μl/min, the stream select valve was switched to the data acquisition position, and the peptides were eluted into the mass spectrometer with an acetonitrile gradient (5%–70% acetonitrile) for 10 min. The capillary voltage was set to 3500 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 500–1000. Tryptsin autolysis products and keratin-derived precursor ions were automatically excluded. The collision voltage was varied between 18 and 45 V depending on the charge and mass of the precursor. Product ion spectra were charge-state de-convoluted and de-isotoped with a maximum entropy algorithm (MaxEnt 3, Micromass). Proteins were identified by the correlation of uninterpreted tandem mass spectra to entries in SwissProt/TREMBL, using ProteinLynx Global Server (Version 1.1, Micromass). One missed cleavage peptide was allowed, and an initial mass tolerance of 50 ppm was used in all searches (27). The search allowed for possible modifications of peptides by iodoacetamide, which if present should identify the cysteine residue(s) involved in disulfide bond formation. Maleimide-containing peptides would identify cysteines not involved in the oxidative associations.

RESULTS

Fig. 1 shows a selection of Coomassie-stained diagonal gels, which were carried out as part of a detailed assessment of interventions that might promote interprotein disulfide formation. Only a selection of the stained gels collected in these studies are shown here. In summary, treatment with hydrogen peroxide (0.1 mM), SNAP (0.01 mM, 0.1 mM or 1 mM), doxorubicin (0.001 mM, 0.01 mM or 0.1 mM), N-acetylcysteine (10 mM), or simulated ischemia (1, 3 or 6 h) failed to increase or decrease interprotein disulfide bonds detected in control preparations. In contrast, diamide (5 mM) and hydrogen peroxide (1 and especially 10 mM) were particularly efficacious at promoting these protein associations, as indicated by spots or bands below and to the left of the diagonal. Spots or bands that run off of the diagonal and to the left of the slope indicate proteins that formed protein-protein disulfides. There was a trend for metabolic inhibition and doxorubicin to initiate some protein interactions, but this was relatively minor compared with diamide (5 mM) and hydrogen peroxide (10 mM) interventions.

We used isolated adult ventricular myocytes under control conditions or following treatment with the thiol oxidant diamide at a concentration of 5, 50, or 100 mM. Visual inspection showed no discernable differences between the cells in any of the test solutions and thus no visible evidence that the cells were particularly stressed. In subsequent studies (see below) in which we assessed intermolecular disulfide formation; cells were incubated in 5 mM diamide for 10 min. On this basis, we
have reasoned that the degree of oxidative stress we imposed on the cells in these studies was mild.

Although diamide treatment caused no visible signs of damage, Fig. 2 illustrates that the cells are still under an oxidative burden. This is illustrated by the fact that myocytes treated with diamide (5 mM for 10 min) promoted S-glutathiolation of several proteins. The signals produced by S-glutathiolated proteins were abolished when 2-mercaptoethanol was added to the samples, confirming that the signal was dependent on the presence of a disulfide bond, which helps confirm the specificity of the antibody.

Fig. 3A is a Coomassie-stained diagonal gel of a cytosolic-enriched fraction. The sample was prepared from control or diamide-treated isolated ventricular myocytes. Clearly some proteins such as peroxiredoxin 2 (Fig. 3A, band 4) run off of the diagonal in control preparations as well, possibly illustrating a disulfide-mediated interaction under basal conditions. However, peroxiredoxin 2 moved further to the left following diamide treatment and formed a weakly stained broad band on the gel, which was clearer on the actual gel and is now highlighted by a dotted outline (Fig. 3A, band 5). Fig. 3B shows a Coomassie-stained diagonal gel of a membrane-enriched fraction, comparing intermolecular protein disulfides in control or diamide-treated cells. As occurred in the cytosolic fraction, a number of membrane proteins underwent diamide-mediated disulfide bonding. Diamide also promoted intermolecular protein disulfide in the Triton-insoluble fraction (Fig. 3C), which is enriched in myofilament, cytoskeletal, and nuclear proteins. Together these diagonal SDS-polyacrylamide gels show widespread protein-protein disulfide bond formation, in multiple subcellular locations, following mild oxidative stress.

Proteins that ran off of the diagonal (i.e. formed protein disulfides) following diamide treatment were identified using LC-MS/MS analysis of tryptic digests. The full list of proteins identified in this way is shown in Table I. We had hoped to map a significant number of the actual cysteine residues involved in interprotein disulfide formation, which are identified by iodoacetamide labeling. However, on the whole this was not successfully achieved. We did observe Cys-289 of acyl-CoA dehydrogenase, Cys-471 of SERCA2, and Cys-145 of the a subunit of the trifunctional enzyme a subunit to be carboxyamidomethylated (modified by iodoacetamide), indicating that these are cysteines involved in disulfide formation.

Following the identification of the adenine nucleotide transporter (ANT) as a protein that undergoes protein disulfide formation using diagonal SDS-PAGE, we analyzed samples under non-reducing or reducing conditions by Western immunoblotting (Fig. 4). ANT formed multiple disulfide-mediated interactions following diamide treatment, although only a relatively small proportion of the total pool of this protein formed these associations. In contrast, when we assessed the distribution of the regulatory subunit of PKA (Fig. 5A), which was also identified using diagonal gels, we found the total pool of this kinase shifted to approximately double its molecular weight via disulfide bond formation. We also assessed the subcellular distribution of this protein (Fig. 5B) and found that the kinase subunit remained primarily in the cytosolic fraction following diamide treatment.

Fig. 6 shows non-reducing immunoblots, which demonstrate that PKC isoforms form interprotein associations following diamide treatment. Treatment of the samples with 2-mercap-
Widespread Intermolecular Protein Disulfide Formation

**FIG. 3.** Coomassie-stained diagonal gels showing widespread disulfide bond formation in diamide (5 mM)-treated cells. A, cytosolic-enriched fraction, the sample was prepared from control or diamide-treated isolated ventricular myocytes. Proteins that run off of the diagonal, to the left represent proteins that formed oxidative disulfide-mediated associations following diamide treatment. B, analysis of the membrane-enriched fraction showed that diamide promoted disulfide-mediated protein interactions in this subcellular compartment as well. C, similarly, an analysis of a Triton-insoluble fraction, which is enriched in myofilament, cytoskeletal, and nuclear proteins, demonstrated that diamide also promoted intermolecular protein disulfide formation at this location.

**DISCUSSION**

**Cellular Redox State in Health and Disease**—Healthy cells are generally found in the reduced redox state, the maintenance of which is dependent on the equilibrium between oxidized and reduced thiols (10, 28). Small thiol-containing molecules (particularly glutathione), as well as protein thiols, are present in high abundance and consequently dominate the cellular redox potential. Some subcellular locations exist in a more oxidized state even under basal conditions, including the endoplasmic reticulum (29) and the extracellular surface (3). Pro-oxidizing events occur in healthy cells following up-regulation of metabolism, receptor activation, and kinase-induced oxidase stimulation (12–14). In addition, oxidative stress has been implicated in the pathogenesis of many diseases, including those of the cardiovascular system (1–5).

**Diagonal Electrophoresis and Detection of Interventions That Induce Protein-Protein Disulfides**—Sequential two-dimensional non-reducing/reducing gel SDS-PAGE (diagonal electrophoresis) has been used previously to investigate proteins that form disulfides with other proteins (21). Rather unexpectedly, the nitric oxide donor SNAP failed to initiate these intermolecular protein disulfides, which we had predicted would take place through mechanisms involving protein S-nitrosylation or S-glutathiolation (which may occur via nitrosoglutathione formation). Simulated ischemia also failed to increase protein-protein disulfides, but this study was complicated by the fact that significant cell death accompanied the longer durations of this intervention. Doxorubicin, which is used in cancer treatment but causes cardiac damage involving oxidative stress, induced a small increase in protein-protein disulfides. In contrast, diamide and hydrogen peroxide were particularly capable of promoting these interactions. Hydrogen peroxide not only contributes to injury in pathologies involving oxidative stress but is also an important mediator of cell signaling (9), and disulfide bond formation may be an important mechanism by which responses to this oxidant are manifested. The diazine carbonyl compound diamide reacts preferentially with reduced glutathione (GSH) compared with other major thiol-containing molecules. At low concentrations (1–10 mM) this compound is tolerated well by biological systems (30), and glutathione disulfide is formed stoichiometrically from two GSH molecules. A predicted consequence of glutathione disulfide accumulation is protein S-glutathiolation via disulfide exchange reactions and is observed on immunoblots probed with the anti-GSH antibody. Protein S-glutathiolation may be an important step in the formation of protein-protein disulfides, although it is diffi-
| Spot number | Protein identified       | Identification code |
|-------------|--------------------------|---------------------|
| 1           | Unidentified             |                     |
| 2           | Nucleoside diphosphate kinase A | NDKA_RAT          |
| 3           | Heat shock 20 kDa-like protein p20 | HSBE_RAT          |
| 4, 5        | Peroxiredoxin 2          | PDX2_RAT           |
| 6           | Unidentified             |                     |
| 7           | Peroxiredoxin 3          | gi 11968132        |
| 8           | Triose-phosphate isomerase | TPI3_RAT          |
| 9–10        | Unidentified             |                     |
| 11          | Skeletal muscle LIM-protein 3 | SLI3_RAT          |
| 14–17       | Glyceraldehyde-3-phosphate dehydrogenase | G3P_RAT         |
| 18          | Actin                    | gi 113290          |
| 19          | Creatine kinase mitochondrial 2 |                  |
| 20          | Actin β-rat              | ACTB_RAT           |
| 21          | cAMP-dependent protein kinase type I al | KAP0_RAT        |
| 22          | Tubulin α-4 chain        | gi 6678467         |
| 22          | Serine/threonine protein phosphatase 2A | 2ABA_RAT         |
| 23          | Tubulin α-4 chain        | gi 6678467         |
| 24          | Similar to tubulin α-1 chain |                  |
| 25          | T-complex protein 1, α subunit | TCPA_RAT         |
| 26          | Unidentified             |                     |
| 27          | HSP 90-β                | HS9B_RAT           |
| 28, 29      | Glycogen phosphorylase   | PHS2_RAT           |
| 30          | Similar to amylo-1, 6-glucosidase, 4-α-glucanotransferase isofrom 1 | gi 34859913    |
| 31          | Superoxide dismutase     | SODC_RAT           |
| 32          | Superoxide dismutase     | SODM_RAT           |
| 34          | Antioxidant protein      | gi 21685578        |
| 34          | Kynureninase             | gi 2143818         |
| 35–39       | ADP, ATP carrier protein | ADT1_RAT           |
| 35–39       | DNA segment, Chr 10     | gi 209070420       |
| 41          | Manganese superoxide dismutase | gi 56691    |
| 42          | ADP, ATP carrier protein | ADT1_RAT           |
| 42          | Phosphate-carrier protein | MPCP_RAT          |
| 42          | Phosphate-carrier protein | MPCP_RAT          |
| 42          | Phosphate-carrier protein | MPCP_RAT          |
| 42          | 3-Hydroxyisobutyrate dehydrogenase precursor | gi 111295    |
| 46          | Glyceraldehyde-3-phosphate dehydrogenase | gi 8393418  |
| 46          | Malate dehydrogenase     | MDHM_RAT           |
| 46          | Glyceraldehyde-3-phosphate dehydrogenase | gi 8393418  |
| 48          | Malate dehydrogenase     | MDHM_RAT           |
| 49          | ADP/ATP transport protein | gi 2143570       |
| 49          | Similar to Peci protein  | gi 34875291       |
| 50          | Aspartate transaminase precursor | gi 90311    |
| 51          | Isocitrate dehydrogenase | gi 27370916       |
| 51          | Acyl coenzyme A thioester hydrolase | MTE1_RAT    |
| 51          | Actin                    | gi 34856664        |
| 51          | 3-Ketoacyl-CoA thiolase  | THIM_RAT           |
| 52          | Creatine kinase          | gi 36289260       |
| 53          | Isocitrate dehydrogenase | gi 27370916       |
| 53          | Elongation factor, Tu   | Q6BFR5             |
| 53          | Similar to RIKEN cDNA   | gi 34859187       |
| 54          | Isocitrate dehydrogenase | gi 27370916       |
| 55          | Tubulin β-15 chain      | gi 92930           |
| 55          | Dihydrolipoamide succinyltransferase comp | ODO2_RAT |
| 55          | Calsequestrin            | CAQ2_RAT           |
| 55          | Similar to sarcalumenin | gi 34868681       |
| 55          | Ac1164                   | gi 32264613        |
| 56          | Sarcolemmal reticulum 55K glycoprotein pre | Q6B563  |
| 56          | Dihydrolipoamide succinyltransferase comp | ODO2_RAT |
| 56          | H(+)-transporting ATP synthase | gi 57029    |
| 57          | ATP synthase α chain    | ATPA_RAT           |
| 57          | Propionyl-CoA carboxylase β chain | PCCB_RAT |
| 58          | Unidentified             |                     |
| 60          | Amine oxidase            | AOFB_RAT           |
| 61          | Dihydrolipoamide acetyltransferase component | ODP2_RAT |
| 61          | Acyl-CoA dehydrogenase, very long-chain s | ACIV_RAT |
| 62          | Succinate dehydrogenase complex, subunit A, flavoprotein | gi 18426858  |
| 63          | Trifunctional enzyme α subunit | ECHA_RAT |
| 63          | Dihydrolipoamide acetyltransferase component | ODP2_RAT |
| 63          | NADH-ubiquinone oxidoreductase | NUAM_HUMAN |
| 64          | Long-chain fatty acid—CoA ligase | LCFB_RAT |
| 64          | Hydroxyacyl-CoA enzyme A dehydrogenase | gi 18677763  |
| 64          | NADH dehydrogenase       | gi 21704020       |
| 65          | Long-chain fatty acid—CoA ligase | LCFB_RAT |

**Table I**

List of proteins that form intermolecular disulfide bonds during myocyte oxidative stress
cult to pinpoint the mechanisms that dominate in this system. Protein-protein disulfides can also form via S-nitrosylation or sulfenic acid formation in a reaction mechanism that involves subsequent reduction by a proximal protein cysteine. However, the nitric oxide donor SNAP did not promote protein-protein disulfide interactions in these studies, indicating that S-nitrosylation may not mediate these events. Consequently, we assessed the protein-protein disulfide formation following diamide treatment in three compartments of the isolated cardiac myocyte by subcellular fractionation, to enhance the resolution of the system and the likelihood of detecting proteins in low abundance. An increase in sensitivity might have been achieved using more sensitive detection methods. However, alternative methods will not prevent highly abundant proteins from dominating the staining pattern, hindering the identification of adjacent proteins in low abundance.

The number of protein-protein disulfides detected was greatly enhanced following diamide treatment. However, a basal level of disulfide-mediated associations was also apparent from examination of the “control diagonal” and may be expected because of a background level of oxidation. This basal level of oxidation also allows for the possibility of functional effects to be coupled to interventions that are proreducing (10, 11). For example, a key event during apoptosis is the export of GSH to the outside of the cell, which is typically an oxidizing environment (3). This decreases the oxidation potential at the external surface of cells, which may promote the reduction of cell surface protein, cysteines, with the potential of a functional correlate.

We have consistently analyzed a control sample directly next to a diamide-treated sample, which reduces difficulties in analysis and interpretation because of intergel differences during electrophoresis or staining. The side-by-side control allows proteins that form interprotein disulfides to be clearly identified, and not misconstrued with proteins that run off of the diagonal for other reasons. Regarding peroxiredoxin 2 (Fig. 3A, band 4), a proportion of this protein migrated even further to the left of the diagonal following diamide treatment, consistent with it interacting with additional proteins via disulfide formation during oxidative stress.

Proteins That Form Interprotein Disulfide—This study has identified a large number of proteins that form intermolecular disulfides. To confirm these as “real” associations, we have also used non-reducing SDS-PAGE and immunoblotting with an antibody to the adenine nucleotide translocator (ANT), a dominant protein found to undergo these oxidative associations, we have also used non-reducing SDS-PAGE and immunoblotting with an antibody to the adenine nucleotide translocator (ANT), a dominant protein found to undergo these oxidative associations.
verify the identifications we have made and validate the diagonal electrophoresis methods for detection of interprotein disulfides.

Future studies might address the effects of these oxidative interactions on the function of the individual proteins. This would require a significant number of studies because of the large number of proteins that participate in these events. It will be a major challenge to integrate the combined effects of the multiple disulfide-mediated associations into an overall functional effect. Furthermore, it is likely that the majority of proteins present in low abundance that form these interactions are yet to be discovered. This idea is supported by the fact that most of the proteins we identified are structural or metabolic enzymes found at high levels in cardiac myocytes. We only identified a few signaling proteins including the regulatory subunit of PKA, nucleoside diphosphate kinase B, and the regulatory subunit of the serine/threonine protein phosphatase 2A, which is consistent with phosphatases having redox active thiols (16). We previously found nucleoside diphosphate kinase B to be an S-thiolation substrate in isolated hearts subjected to ischemia and reperfusion (15, 32). However, this enzyme regulates nucleoside diphosphate levels and is not involved in signaling via protein phosphorylation. When we assessed PKA distribution on non-reducing Western blots, we found that the total pool of this protein shifts to a molecular weight consistent with its dimerization. This observation has been described previously (33) and accounts for the oxidation-dependent inhibition of this protein. Both troponin I and SERCA2 formed disulfide associations, which could have implications for the modulation of cardiac excitation contraction coupling by oxidant stress via interprotein disulfide bond formation.

Previously we found PKC to be redox-active during cardiac oxidative stress (15, 32). We reasoned that they may not have been detected using diagonal electrophoresis because of a relative low abundance, and so we assessed PKC intermolecular disulfides using immunoblotting. We found that PKCγ, PKCe, and, to a lesser extent, PKCa formed protein disulfide complexes following diamide treatment. However and in contrast, using the same immunoblotting approach we did not observe that HSP60 or HSP70 formed interprotein disulfides. This is despite our group and others (15, 34–36) having found heat shock proteins to have redox active thiols. This observation highlights the potential for proteins with redox active thiols to undergo specific or preferential forms of oxidative modification, which lends itself to the possibility of differential functional effects depending on the exact mode of cysteine oxidation.

In addition to discrete spots (which indicate interactions with single proteins), a number of horizontal bands or stripes of
Widespread Intermolecular Protein Disulfide Formation

PKC α

PKC δ

PKC ε

FIG. 6. Non-reducing and reducing immunoblotting shows that PKC isoforms form interprotein associations following diamide treatment, which results in multiple immunoreactive bands migrating at a higher molecular weight (Mwt). However, when these samples were treated with 2-mercaptoethanol, the banding pattern was normalized. This shows that the diamide-induced PKC associations involve the formation of intermolecular protein disulfide bonds.

Conclusions and Potential Consequences of Interactions Discovered—A significant number of proteins with diverse functions have been shown in this study to form intermolecular disulfides following oxidative stress in isolated cardiac myocytes. A full consideration of the potential functional consequences for each of the proteins involved would take considerable space and is beyond the scope of this text. However, it should be noted that many of the proteins that have been identified make sense, as they are already known to have redox active thiols. For example, we have identified many of them as S-thiolation substrates in tissues under oxidative stress (15, 40, 41). A number of the proteins identified here were also recently found to be S-nitrosylation substrates (42).

Currently there is a growing awareness of the importance of oxidants as signaling molecules that directly impact the function of tissues in a regulatory non-damaging way by altering the structure of proteinaceous cysteinyl thiols. Multiple modes of protein-cysteine oxidation, such as S-thiolation (15), S-nitrosylation (8), sulfenic acid (16), sulfenic acid (17), and sulfinyl-amide formation (18), are already known to vary degrees to be important in redox regulation. This study shows that intermolecular protein disulfide formation is a widespread phenomenon involving a great many proteins, illustrating the potential of these oxidative associations in the redox regulation of cell function.

REFERENCES

1. Klein, J. A., and Ackerman, S. L. (2003) J. Clin. Investig. 111, 785–793
2. Finkel, T., and Holbrook, N. J. (2000) Nature 408, 239–247
3. Skulachev, P. V., Hedges, B., Klenchin, N. A., and Herzenberg, L. A. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 4001–4005
4. Kono, H., Rusyn, I., Yin, M., Gábele, E., Yamashina, S., Dikalova, A., Kadiiska, M. B., Conner, H. D., Mason, R. P., Segal, B. H., Bradford, B. U., Holland, S. M., and Thurman, R. G. (2000) J. Clin. Investig. 106, 867–872
5. Griendling, K. K., and FitzGerald, G. A. (2003) Circulation 108, 1912–1916
6. Soberman, R. J. (2003) J. Clin. Investig. 111, 571–574
7. Finkel, T. (1998) Curr. Opin Cell Biol. 10, 248–253
8. Stamlar, J. S., Lamas, S., and Fang, F. F. (2001) Cell 106, 675–683
9. Rhee, S. G., Bae, Y.-S., Lee, S.-R., and Kwon, J. (2000) Science's STKE www.stke.org/cgi/content/full/OC_sigtrans/2000/53/pe1
10. Schaf, F. Q., and Bueettner, G. R. (2001) Free Radic. Biol. Med. 30, 1191–1212
11. Chandra, J., Samali, A., and Orrenius, S. (2000) Free Radic. Biol. Med. 29, 323–333
12. Rhee, S. G. (1999) Exp. Mol. Med. 31, 53–59s
13. Mahadev, K., Wu, X., Zilbering, A., Zhu, L., Lawrence, T. R., and Goldstein, B. J. (2001) J. Biol. Chem. 276, 48662–48669
14. Lopez-Ongil, S., Senchak, V., Saura, M., Zagarza, C., Ames, M., Ballermann, B., Rodriguez-Puyol, M., Rodriguez-Puyol, D., and Lowenstein, C. J. (2000) J. Biol. Chem. 275, 26423–26427
15. Eaton, P., Hyers, H. L., Leeds, M. A., and Shattock, M. J. (2002) J. Biol. Chem. 277, 8906–8911
16. Poole, L. B., Karplus, P. A., and Claiborne, A. (2004) Annu. Rev. Pharmacol. Toxicol. 44, 325–347
17. Bitezou, B., Labarre, J., and Toledano, M. B. (2003) Nature 425, 980–984
18. Salmen, A., Andersen, J. N., Myers, M. P., Meng, T.-C., Hinks, J. A., Tonks, N. K., and Barford, D. (2001) Nature 425, 769–773
19. Eaton, P., Hearse, D. J., and Shattock, M. J. (2001) Cardiovasc. Res. 52, 294–301
20. Gittler, C., Zarni, B., and Kofel, E. (1997) Anal. Biochem. 252, 48–55
21. Sumner, A., and Rattan, S. R. (1974) Proc. Natl. Acad. Sci. U. S. A. 71, 3946–3950
22. James, A. F., Ramsey, J. E., Reynolds, A. M., Hendry, B. M., and Shattock,
Widespread Intermolecular Protein Disulfide Formation

M. J. (2001) Biochem. Biophys. Res. Commun. 284, 1048–1055
23. Jeno, P., Mini, T., Moes, S., Hintermann, E., and Horst, M. (1995) Anal. Biochem. 224, 75–82
24. Wilm, M., Shevchenko, A., Houthaeve, T., Breit, S., Schweigerer, L., and Fotsis, T. (1996) Nature 379, 466–469
25. Shevchenko, A., Wilm, M., Vorm, O., and Mann, M. (1996) Anal. Chem. 68, 850–858
26. Wait, R., Gianazza, E., Eberini, I., Sironi, L., Dunn, M., Gemeiner, M., and Miller, I. (2001) Electrophoresis 22, 3043–3052
27. Wait, R., Miller, I., Eberini, I., Cairoli, F., Veronesi, C., Battochio, M., Gemeiner, M., and Gianazza, E. (2002) Electrophoresis 23, 3418–3427
28. Meister, A., and Anderson, M. E. (1983) Annu. Rev. Biochem. 52, 711–760
29. Tu, B. P., and Weissman, J. S. (2004) J. Cell Biol. 164, 341–346
30. Sies, H. (1985) in Oxidative Stress (Sies, H., ed) pp. 73–90, Academic Press, London
31. Baines, C. P., Song, C. X., Zheng, Y. T., Wang, G. W., Zhang, J., Wang, O. L., Guo, Y., Bolli, R., Cardwell, E. M., and Ping, P. P. (2003) Circ. Res. 92, 873–880
32. Eaton, P., and Shattock, M. J. (2001) Circulation 104, 290
33. Humphries, K. M., Juliano, C., and Taylor, S. S. (2002) J. Biol. Chem. 277, 43555–43561
34. Eaton, P., Fuller, W., and Shattock, M. J. (2002) J. Biol. Chem. 277, 21189–21196
35. Fratelli, M., Demol, H., Puype, M., Casagrande, S., Eberini, I., Salmona, M., Bonetto, V., Mengozzi, M., Duffieux, F., Miclet, E., Bachi, A., Vandekerckhove, J., Gianazza, E., and Ghezzi, P. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 3505–3510
36. Lind, C., Gerdes, R., Hamnell, Y., Schuppe-Keistinen, I., von Lowenhielm, H. B., Holmgren, A., and Cotgreave, I. A. (2002) Arch. Biochem. Biophys. 408, 226–240
37. Cumming, R. C., Andon, N. L., Haynes, P. A., Park, M., Fischer, W. H., and Schubert, D. (2004) J. Biol. Chem. 279, 21749–21758
38. Hampton, M. B., Kettle, A. J., and Winterbourn, C. C. (1998) Blood 92, 3007–3017
39. Marcella, C., Neverova, I., Menaho, R., Van Eyk, J., and Di Lisa, F. (2004) Am. J. Physiol. 286, H870–H877
40. Eaton, P., and Shattock, M. J. (2002) Ann. N. Y. Acad. Sci. 973, 1–4
41. Eaton, P., Jones, M. E., McGregor, E., Dunn, M. J., Leeds, N., Byers, H. L., Leung, K. Y., Ward, M. A., Pratt, J., and Shattock, M. J. (2003) J. Am. Soc. Nephrol. 14, S280–S286
42. Kuncewicz, T., Sheta, E. A., Goldknopf, I. L., and Kone, B. C. (2003) Mol. Cell Proteomics 2, 156–163
43. National Academy of Sciences (1996) Guide for the Care and Use of Laboratory Animals, National Academy Press, Washington, D. C.
Detection and Mapping of Widespread Intermolecular Protein Disulfide Formation during Cardiac Oxidative Stress Using Proteomics with Diagonal Electrophoresis
Jonathan P. Brennan, Robin Wait, Shajna Begum, James R. Bell, Michael J. Dunn and Philip Eaton

J. Biol. Chem. 2004, 279:41352-41360.
doi: 10.1074/jbc.M403827200 originally published online August 2, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M403827200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 40 references, 15 of which can be accessed free at
http://www.jbc.org/content/279/40/41352.full.html#ref-list-1