Protein disulfide bond formation in the cytoplasm during oxidative stress

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Running Title: Disulfide bond formation in the cytoplasm
Summary

The majority of disulfide-linked cytosolic proteins are thought to be enzymes that transiently form disulfide bonds while catalyzing oxidation-reduction (redox) processes. Recent evidence indicates that reactive oxygen species (ROS) can act as signaling molecules by promoting the formation of disulfide bonds within or between select redox sensitive proteins. However, few studies have attempted to examine global changes in disulfide bond formation following ROS exposure. Here we isolate and identify disulfide-bonded proteins (DSBP) in a mammalian neuronal cell line (HT22) exposed to various oxidative insults by sequential nonreducing/reducing two-dimensional SDS-PAGE (Redox 2D-PAGE) combined with mass spectrometry. Using this strategy, several known cytosolic DSBP, such as peroxiredoxins, thioredoxin reductase, nucleoside diphosphate kinase and ribonucleotide diphosphate reductase, were identified. Unexpectedly, a large number of previously unknown DSBP were also found, including those involved in molecular chaperoning, translation, glycolysis, cytoskeletal structure, cell growth and signal transduction. Treatment of cells with a wide range of hydrogen peroxide concentrations either promoted or inhibited disulfide bonding of select DSBP in a concentration-dependent manner. Decreasing the ratio of reduced to oxidized glutathione also promoted select disulfide bond formation within proteins from cytoplasmic extracts. In addition, an epitope-tagged version of the molecular chaperone Hsp70 forms mixed disulfides with both β4-spectrin and adenomatous polyposis coli protein in the cytosol. Our findings indicate that disulfide bond formation within families of cytoplasmic proteins is dependent on the nature of the
oxidative insult and may provide a common mechanism used to control multiple physiological processes.
Introduction

Oxidative stress occurs when the rate of reactive oxygen species (ROS)\(^1\) generation exceeds the detoxification abilities of the cell, and it has been implicated in many degenerative diseases. It is frequently argued that ROS cause relatively non-specific damage to vital cellular components such as lipids, DNA and proteins. However, emerging evidence indicates that ROS can cause specific protein modifications that may lead to a change in the activity or function of the oxidized protein (1, 2). Several major forms of oxidative modifications can occur on amino acid residue side chains including carbonylation, nitrosylation and oxidation of methionine to methionone sulfoxide (3). Protein sulfhydryls can be oxidized to protein disulfides and sulfenic acids as well as more highly oxidized states such as the sulfinic and sulfonic acid forms of protein cysteines (4). Under non-stressed conditions, disulfide bond formation occurs primarily in the oxidizing environment of the endoplasmic reticulum (ER) in eukaryotic cells (5). The sulfhydryl groups in the vast majority of protein cysteine residues (Cys-SH) have a pKa>8.0 and, in the reducing environment of the cytoplasm, remain protonated at physiological pH. Thus, cytoplasmic proteins, in general, do not contain disulfide bonds (6). However, certain redox sensitive proteins possess cysteine residues that exist as thiolate anions at neutral pH due to a lowering of their pKa values by charge interactions with neighboring amino acid residues and are therefore more vulnerable to oxidation (6).

\(^1\) The abbreviations used are: ROS, reactive oxygen species; GSH, reduced glutathione, GSSG, oxidized glutathione; Prot-SSG, protein mixed disulfide; DSBP, disulfide-bonded proteins; IA, iodoacetamide; BIAM, biotin-conjugated iodoacetamide; TRX, thioredoxin; Prx 1, peroxiredoxin 1; NDPK-B, nucleoside diphosphate kinase B; H\(_2\)O\(_2\), hydrogen peroxide; Redox 2D-PAGE, nonreducing/reducing two-dimensional polyacrylamide gel electrophoresis; MALDI-TOF, matrix-assisted laser desorption/ionization time-or-flight; MS/MS, tandem mass spectrometry.
Many redox sensitive proteins form transient disulfide bonds while catalyzing the reduction of thiol groups (6). There are two major thiol reducing systems in the cytoplasm. The first system makes use of the abundant cysteine-containing tripeptide glutathione (GSH) to reduce disulfide bonds via a thiol-disulfide interchange catalyzed by glutaredoxin (7). In the second system, the reduced form of thioredoxin (TRX) binds to substrate proteins containing a disulfide bond, and a dithiol-disulfide exchange reaction occurs in which the active site cysteine residues of TRX are oxidized while the cysteine residues in the substrate protein are reduced (8). During severe oxidative stress both systems can be overwhelmed or inactivated, leaving cytosolic cysteine residues susceptible to oxidation.

Recently, a number of studies in bacteria and yeast have shown that oxidative stress-induced disulfide bond formation appears to be the main mechanism to adjust the protein activity of the transcription factors OxyR and Yap1 and the molecular chaperone Hsp33 (reviewed in 9). Reversible oxidation of redox sensitive proteins has also been shown to regulate signal transduction and gene expression in mammalian cells (reviewed in 10). Hydrogen peroxide (H$_2$O$_2$), a relatively mild oxidant implicated in both oxidative stress and cell signaling, can oxidize cysteine sulfhydryl groups to a cysteine sulfenic acid (Cys-SOH) or disulfide bonds (4). The active site cysteine residue of protein tyrosine phosphatases (PTPs) has been shown to be reversibly oxidized to a sulfenic acid by H$_2$O$_2$ (11, 12). In addition, TNF-α-induced signaling generates ROS that promote the oxidation of the active site residues of TRX. Oxidation of TRX results in its dissociation from apoptosis signal-regulating kinase 1 (ASK1) and the subsequent activation of ASK1 (13). Thus, an increase in intracellular ROS as a
result of exposure to environmental stimuli including cytokines, growth factors, radiation and chemical agents, can lead to oxidation of cysteine residues within cytoplasmic redox sensitive proteins, such as kinases and phosphatases, ultimately affecting signal transduction processes. However, the actual number and type of redox sensitive proteins which undergo reversible cysteine oxidation is, for the most part, unknown.

Relatively few methods exist for examining disulfide bond formation within cells. A technique termed diagonal SDS-PAGE was originally used to analyze intermolecular disulfide bonds artificially generated between ribosomal proteins in E. coli (14). In diagonal SDS-PAGE, oxidized proteins are separated in the first dimension by electrophoresis under non-reducing conditions and in the second dimension under reducing conditions. This technique has successfully been adapted to monitor mixed disulfide bond formation during the folding of newly synthesized proteins in the ER (15, 16). Using a well characterized neuronal cell model of oxidative stress, we isolated and identified cytosolic DSBP based on a modified diagonal 2D-PAGE method and mass spectrometry. We show here that protein disulfide bond formation readily occurs within the cytoplasm of unstressed as well as oxidant-stressed cells and that disulfide bond formation is dependant on the type of oxidant exposure. These redox sensitive proteins, previously not known to form disulfide bonds, participate in numerous cellular processes including translation, molecular chaperoning, glycolysis, cell growth, cytoskeletal structure, antioxidant activity and signal transduction, suggesting that disulfide bond formation may have a regulatory role in these processes.
Materials and Methods

Cell lines and oxidant exposure—HT22 cells (17) were grown in DMEM containing 10% FCS. Cells were maintained at no greater than 50% confluence. For oxidant exposure studies, 2.5 X 10^5 cells were seeded per 100mm dish and the following day either exposed to 5 mM glutamate for 8 hrs, 1 mM diamide for five minutes or 10 mM H2O2 for five minutes. In some studies cells were exposed to either 10, 150 or 400 µM H2O2 for 10 minutes.

Glutathione measurement—Cells were washed twice with PBS, harvested in 1% sulfosalicylic acid and centrifuged at 16,000 x g for 10 minutes. Glutathione and protein assays were performed on the supernatant and pellet respectively. Total glutathione was measured by a standard recycling assay based on the reduction of 5,5-dithiobis-2-nitrobenzoic acid in the presence of glutathione reductase and NADPH (18). GSSG was separately measured after derivatization of GSH with 2-vinylpyridine. Prot-SSG levels were measured after sonicating and rinsing the protein pellets in 1% sulfosalicylic acid then resuspending the pellet in 0.01 M Tris-Hcl. pH 7.5. The solution was then treated for 45 min at 41°C with 0.25% sodium borohydride at neutral pH to reduce the disulfide linkage. Excess borohydride was decomposed by acidification and the liberated GSH was measured as described above.

Cytosolic fractionation and protein precipitation.—Following exposure to various oxidative stimuli, approximately 5 X 10^6 HT22 cells were washed twice with PBS and then incubated in ice cold PBS with 40 mM iodoacetamide (IA) for 5 minutes to prevent thiol-disulfide exchange and inhibit post-lysis oxidation of free cysteines. Using a modified differential detergent fractionation technique (19), cells were
scraped, pelleted and resuspended in 0.5 ml digitonin extraction buffer (10 mM PIPES, pH 6.8, 0.015% (w/v) digitonin, 300 mM sucrose, 100 mM NaCl, 3 mM MgCl₂, 5 mM EDTA, 1mM PMSF) + 40 mM IA, followed by rocking on ice for 10 minutes. Following centrifugation at 480xg, the supernatant (cytosolic fraction) was transferred to a new tube and proteins were precipitated with trichloroacetic acid (10% final concentration). The precipitated proteins were resolublized in 100 µl of solublization buffer (100 mM Tris, pH 7.5, 2% SDS, 1mM PMSF) + 40 mM IA.

For membrane/organelle and nuclear fractionation, post digitonin-extracted pellets were further processed using the differential detergent fractionation technique (19).

Redox 2D gel electrophoresis—An equal volume of SDS sample buffer, free of reducing agents, was added to resolublized cytosolic extracts. Gels (10% acrylamide, 1.0 mm thickness) were prepared according to Laemmli (20) and protein samples (120 µg) were subjected to electrophoresis in the first dimension for 5 h at constant current (25 mA) using a Hoefer SE 600 gel apparatus (Amersham Pharmacia Biotech, Piscataway, NJ). After electrophoresis, the gel lanes containing the separated proteins were cut and immersed in SDS sample buffer containing 100 mM DTT for 20 min at room temperature. Following a brief wash with SDS running buffer, the gel slices were further immersed in SDS sample buffer containing 100 mM IA for 10 min. Each gel strip was then applied horizontally to another gel (10% acrylamide, 1.5 mm thickness), and electrophoresis was performed in the second dimension for 14 h at constant current (10 mA/gel). Gels were fixed in 50% methanol, 5 % acetic acid for 20 min and either silver stained according to the method of Schevchenko et al. (21) or stained with GelCode Blue according to the manufacturer (Pierce, Rockford, IL).
Identification of redox sensitive proteins—Gel spots from redox 2D gels were excised and in-gel digested with trypsin (21). The resulting peptides were analyzed by either liquid chromatography-electrospray tandem MS (MS/MS) or matrix-assisted laser-desorption ionization MS (MALDI-TOF). For MS/MS analysis, peptides were initially separated using a microbore HPLC system (Surveyor, ThermoFinnigan, San Jose, CA) and the eluent from the HPLC column was eluted directly into the electrospray ionization source of a ThermoFinnigan LCQ-Deca ion trap mass spectrometer (ThermoFinnigan, San Jose, CA) as described previously (22). MS/MS data were analyzed using the SEQUEST algorithm and positive sequence identifications were based on established criteria such as a cross-correlation factor (Xcorr) greater than 2.5, a delta cross-correlation factor (ΔXcorr) greater than 0.1 and a minimum of one tryptic peptide terminus. All matched peptides were confirmed by visual examination of the spectra. All spectra were searched against a composite database containing the latest version of the non-redundant protein database SWISSPROT. For MALDI-TOF analysis, aliquots of digest samples (0.5 µl) were mixed with an equal volume of matrix solution (saturated solution of α-cyano-4-hydroxycinnamic acid in 0.3% trifluoroacetic acid and 30% acetonitrile) on the target plate. MALDI-TOF spectra were acquired using a Voyager DE-STR instrument (Applied Biosystems, Foster City, CA). The machine employs a nitrogen laser (337 nm) and was run in positive-ion reflector mode with an ion extraction delay. Monoisotopic peptide masses were determined after internal calibration and searched against the NCBInr mouse protein database using the Protein Prospector (UCSF) program with a mass tolerance of 25 ppm.
**Immunoblot Analysis**—Fractionated cytosolic, membrane/organelle and nuclear extracts (15 µg) were resolved using 4-12% nonreducing SDS-PAGE (Novex, Carlsbad, CA), electroblotted onto Immobilon P membrane (Millipore, Bedford, MA) and blocked with 1% milk, 3% BSA in TBS. Blots were hybridized with antibodies against PRX-1, NDPK-B (Santa Cruz Biotechnology Inc., Santa Cruz, CA), Hsc70, Hsp70, PDI (Stressgen, Victoria, BC, Canada), GAPDH (Chemicon, Temecula, CA), actin, α-tubulin (Sigma, St. Louis, MO) and FLAG (IBI/Kodak, New Haven, CT) overnight and, following washing, were further hybridized with appropriate HRP-conjugated secondary antibodies (Biorad, Hercules, CA). Detection was performed using ECL western blotting detection reagents (Amersham Pharmacia Biotech, Piscataway, NJ).

**Induction of disulfide bonding by GSSG**—Cytoplasmic extracts were prepared as described above except IA was not added during harvesting or lysis. Extracts (250 µg) were incubated for 1 hour at room temperature in the presence of different ratios of GSH and GGSG. The total concentration of GSH equivalents was maintained at 5 mM in the incubation mix. Following incubation, lysates were precipitated with 10% trichloroacetic acid, solubilized and analyzed by Redox 2D-PAGE as described above.

**Labeling of DSBP with biotin-conjugated iodoacetamide**—Proteins were first separated by nonreducing gel electrophoresis, then the gel lanes were cut and immersed in 25 mM Tris pH 7.0, 1 mM EDTA and 100 mM DTT. Following in-gel reduction for 20 minutes gel slices were washed three times in Tris/EDTA buffer and incubated with 50 µM N-(Biotinoyl)-N’-(iodoacetyl)-ethylenediamine (Molecular Probes) in 25 mM Tris pH 6.5, 0.1 mM EDTA for 20 minutes at room temperature.
Gel slices were applied horizontally to another gel and electrophoresis was performed as described above followed by electroblotting onto PVDF membrane and detection using streptavidin-conjugated horse radish peroxidase (Roche, Indianapolis IN).

**Construction of epitope-tagged Hsp70 vector**—Human Hsp70 cDNA was amplified by polymerase chain reaction using the primer sets, 5’-GGCGGATCCACCATGGCCAAAGCCGCAGTC-3’ and 5’-TGCGGTCGACTCTATCGTCGTCATTGTAATCATCTACCTCCTCAATGGTGGG-3’ and pH2.3/Hsp70 (kindly provided by Richard I. Morimoto, Northwestern University, Evanston, IL) as a template. BamH1 and Sal1/FLAG sequences were incorporated into the 5’ and 3’ primers respectively. The PCR product was digested with BamH1 and Sal1, inserted into a BamH1/Sal1 cut pRevTRE vector (Clontech, Palo Alto, CA) and sequenced to verify the fidelity of the PCR reaction and ensure that the FLAG sequence was incorporated. The resulting vector (Hsp70-Flag) was transfected into HT22 cells expressing the reverse tetracycline-controlled transactivator. Expression of Hsp70-Flag was induced by the addition of 2µg/ml doxycycline for 24 hrs.

**Immunoprecipitation**—Cytosolic extracts were prepared as described above and precipitated with trichloroacetic acid. Acid-precipitated proteins were resolublized in 100 µl of MNT buffer (20 mM MES, 100 mM NaCl, 30 mM Tris-HCl pH 7.5, 1mM EDTA, 1mM PMSF, 0.5% Triton X-100, 20 mM IA) containing 1% SDS. After adjusting the volume to 1 ml with MNT buffer, 20 µl of agarose-coupled M2 anti-Flag antibody (Sigma, St. Louis, MO) was added and incubated at 4°C overnight. After three washes with MNT buffer, the immunocomplexes were competitively
eluted by the addition of 100 µl of FLAG peptide (400 µg/ml). Elution fractions were analyzed by both immunoblotting and Redox 2D-PAGE as described above.
Results

H$_2$O$_2$ and diamide induce protein mixed disulfides with glutathione

We examined the effect of a variety of oxidative conditions on protein disulfide bond formation using the murine hippocampal cell line HT22. This cell line has been used extensively to examine oxidative stress-induced cell death (23-26). Non-receptor mediated oxidative glutamate toxicity is initiated by high concentrations of extracellular glutamate that prevents cystine uptake into cells, which in turn depletes both intracellular cysteine and GSH, and leads to a dramatic increase in ROS and ultimately cell death (23, 24). This form of oxidative stress induced programmed cell death is called oxytosis (27). HT22 cells are highly sensitive to extracellular glutamate, H$_2$O$_2$ and agents that deplete GSH (23, 24). H$_2$O$_2$ is a diffusible mild oxidant that readily promotes the conversion of sulfhydryl groups into disulfides and other oxidized species (10). Diamide is an oxidizing agent that easily penetrates cell membranes and rapidly reacts with low molecular weight thiols (such as GSH) and promotes intracellular protein disulfide cross-linking (28). In eukaryotic cells, cysteine residues within cytoplasmic proteins are maintained in a reduced state due to the high concentration of GSH (2-10 mM) and the high ratio (100-400/1) between GSH and GSH disulfide (GSSG) (29). During oxidative stress the levels of GSH decrease, GSSG levels increase and mixed disulfides form between GSH and redox sensitive proteins (a process termed glutathionylation), all of which are theorized to promote protein disulfide bond formation (29). We therefore examined levels of GSH, GSSG and Prot-SSG in oxidant-treated HT22 cells to verify that the oxidants used would promote disulfide bond formation. As expected, glutamate treatment caused a massive decrease in
GSH levels (Fig. 1A). Although H$_2$O$_2$ treatment had little effect on GSH levels, GSSG levels were increased over three-fold compared to untreated cells (Fig. 1B). In contrast, diamide treatment caused a modest decrease in GSH levels and essentially had no effect on GSSG levels. However, the ratio of GSH/GSSG was 230, 21.3, 78 and 163.2 in control, glutamate, H$_2$O$_2$ and diamide treated cells respectively, indicating that all three treatments promoted oxidation to varying degrees. Interestingly, both H$_2$O$_2$ and diamide treatment caused between a 14 to 19 fold increase in Prot-SSG levels (Fig. 1C).

**Isolation of disulfide-linked proteins in oxidatively challenged HT22 cells by nonreducing/reducing two-dimensional SDS gel electrophoresis**

Following oxidant treatment, cytoplasmic extracts from HT22 cells were isolated using a modified differential detergent fractionation technique (19). To ensure that the cytoplasmic fractions were not contaminated with proteins from either the membrane/organelle or nuclear fractions, we performed immunoblot analysis with antibodies that recognize endoplasmic reticulum and nuclear specific proteins. Essentially no contamination with proteins from the endoplasmic reticulum (protein disulfide isomerase) or the nucleus (CREB) was observed in the cytoplasmic fraction (Fig. 2A).

To isolate proteins that form either intra- or intermolecular disulfide bonds, we sequentially resolved cytosolic proteins by non-reducing followed by reducing SDS-PAGE (Redox 2D-PAGE). The resulting silver-stained gels reveal a prominent diagonal line of proteins (Fig. 2B). This line represents the majority of proteins within the cytosol that do not form disulfide bonds. However, proteins that form intermolecular disulfide bonds exhibit a slower electrophoretic mobility under
non-reducing conditions in the first dimension and therefore appear as spots to the right of the diagonal line following reducing SDS-PAGE in the second dimension. Spots that appear to the left of the diagonal line represent proteins that exhibit a faster electrophoretic mobility under non-reducing conditions due to intramolecular disulfide bonding. In order to verify that acrylamide did not artificially induce disulfide bonding, cytosolic protein extracts were treated with the reducing agent dithiothreitol (DTT) in both the first and second dimension. As expected, the presence of reducing agents in both dimensions resulted in a clear diagonal line with essentially no spots in the off-diagonal zone (Fig. 2F). We treated HT22 cells with concentrations of glutamate, H$_2$O$_2$ and diamide that elicit an oxidative response but lead to less than 10% cell death within the time frame of the experiment. Under non-stressed conditions, numerous disulfide-bonded proteins (DSBP) were apparent (Fig. 2B). Exposure to either glutamate, H$_2$O$_2$ or diamide resulted in increased levels of existing DSBP and the appearance of new DSBP that were not apparent in the non-stressed control cells (Fig. 2C,D,E).

**Identification of DSBPs by mass spectrometry**

Protein spots that exhibited reproducible migration following Redox 2D-PAGE were excised, in-gel digested with trypsin and identified by either matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) or by capillary liquid chromatography-tandem mass spectroscopy (MS/MS). A representative Redox 2D-PAGE gel of cells treated with diamide, that contains all the DSBP spots identified, is presented along with the corresponding spot numbers in Figure 3. Analysis of the DSBP identified revealed that most of the proteins could be classified into the following eight functional categories: molecular chaperones,
protein translation, glycolysis, cytoskeletal, cell growth, antioxidant, signal transduction and other. Table 1 summarizes the DSBP identified and their corresponding spot identification number on Figure 3. Some proteins, such as Hsc70, Hsp70 and Hsp90, formed more than one intermolecular disulfide bond and therefore appeared as several spots to the right of the diagonal line (Fig. 3B). The translation elongation factor, EF-1-alpha-1 formed both intra- and intermolecular disulfide bonds and thus appeared as several spots to the left and right of the diagonal line respectively (Fig. 3A). In some cases, such as spots 1, 5 and 11, multiple proteins were identified per spot (Table 1) due to similar migration patterns in the non-reducing first dimension.

**Verification of DSBP by non-reducing SDS-PAGE and immunoblotting**

The formation of disulfide bonds is known to affect the conformation and electrophoretic mobility of redox sensitive proteins (30-32). Proteins that form intermolecular disulfide bonds exhibit slower migration, whereas proteins forming intramolecular disulfide bonds exhibit faster migration following separation by nonreducing SDS-PAGE compared to the same proteins resolved under reducing conditions. In order to verify that the proteins we identified by Redox 2D-PAGE were bona fide DSBP, we examined cytoplasmic fractions by non-reducing SDS-PAGE and immunoblot analysis using antibodies that specifically recognized the proteins of interest. Spot 30 was determined to be Peroxiredoxin I (Prx I), an enzyme that forms reaction intermediates containing intermolecular disulfide bonds between Prx subunits (33). Furthermore, in the presence of high levels of H$_2$O$_2$, one of the catalytic cysteine residues of Prx I undergoes hyperoxidation to a cysteine-sulfinic acid and is unable to reform a disulfide bond (33, 34).
expected, the Prx I disulfide-linked dimer exhibited slower migration under nonreducing conditions (Fig. 4A). However, following H$_2$O$_2$ treatment, the Prx I dimer shifted to a monomeric form and exhibited migration similar to lysates treated with the reducing agent DTT (Fig. 4A) and no longer appeared as a spot in Redox 2D-PAGE analysis (Fig. 2C).

Spot 32, appearing only in H$_2$O$_2$ and diamide treated cells (Fig. 2C,D), was identified as nucleoside diphosphate kinase (NDPK-B), an enzyme involved in maintaining cellular nucleoside diphosphate levels, and has previously been shown to form disulfide bonds following exposure to either H$_2$O$_2$ or diamide (35). As expected, when HT22 cells were treated with either H$_2$O$_2$ or diamide we observed a disulfide-linked dimer of NDPK-B that dissociated into a monomer under reducing conditions (Fig. 4B).

Heat shock proteins participate in the folding of nascent polypeptide chains in addition to modulating various signal transduction pathways (36). Mass spectrometry analysis revealed that spots 6,7 and 8 in Fig. 2 were Hsc70 and Hsp70. Hsc70 is a constitutively expressed heat shock protein whereas Hsp70 is an induced isoform. Under nonreducing conditions, the levels of several higher molecular weight redox conformers of Hsp70 were slightly reduced after exposure to H$_2$O$_2$ and diamide compared to control and glutamate treated cells (Fig. 4C). In contrast, the level of a 140 kDa redox conformer of Hsc70 was increased after oxidant exposure, particularly after diamide treatment (Fig. 4D). Therefore, depending on the isoform of Hsp70, either an increase or a decrease in disulfide-linked conformers is observed after oxidant exposure.
Spots 15 and 28 (Fig 3A) were identified as glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and α−tubulin respectively (Table 1). Both proteins possess thiol groups susceptible to oxidation (37, 38). Following H₂O₂ and diamide exposure, both proteins formed multiple disulfide linked species, including extremely high molecular weight polymers found in the stacking layer (Fig. 4E,F).

**Decreasing the ratio of GSH to GSSG promotes protein disulfide bond formation**

The formation of intra- and intermolecular disulfide bonds within the ER is a crucial step in the folding of many secretory proteins (39). A glutathione redox buffer (a mixture of GSH and GSSG at a ratio between 1:1 and 3:1) maintains the redox state of the ER more oxidizing than that of the cytosol to allow disulfides to form and rearrange (39). *In vitro* assays for oxidative protein folding typically employ GSSG as an electron acceptor for the oxidation of protein thiols to disulfide bonds. Since H₂O₂ and diamide treatment both promoted increased glutathionylation (Fig 1) and generated similar DSBP profiles (Fig 2) we asked if GSSG itself promotes disulfide bond formation within the cytosol. Given its physiological relevance, we chose the GSH/GSSG redox pair to establish a gradient of oxidative stress. Cytosolic extracts were incubated in the presence of different GSH/GSSG ratios (10:1-1:10) but the total amount of GSH equivalents was maintained at 5 mM. Cytoplasmic extracts incubated with 5 mM GSH alone displayed a pronounced reduction in disulfide bonding (Fig 5A) compared to non-treated extracts (Fig 2B). As the ratio of GSH/GSSG decreased, several DSBP exhibited increased disulfide bonding, including calreticulin, Hsp70, tcp-1β, Bcd orf2, GAPDH and peroxiredoxin 1 (Fig 5B,C,D). Thus, disulfide bonding within a
select group of cytosolic DSBP appears to be promoted by increased levels of GSSG.

**H$_2$O$_2$ concentration-dependent effects on disulfide bonding within cytoplasmic proteins**

In our initial studies, high concentrations of H$_2$O$_2$ (1 to 10 mM) were used to detect changes in disulfide-linked proteins by silver staining (Fig 2D). Although these H$_2$O$_2$ levels can be produced during the acute oxidative burst in neutrophil mediated phagocytosis (40), the effects of lower concentrations of H$_2$O$_2$ (10 to 400 µM) on disulfide bond formation were also examined since these concentrations are more physiologically relevant (41). To detect less abundant DSBP, disulfide bonded proteins were first labeled with biotin-conjugated iodoacetamide (BIAM), separated by Redox 2D-PAGE, electroblotted onto PVDF membrane and finally detected with streptavidin-conjugated HRP. As observed with silver stained proteins (Fig 2D), the majority of BIAM-labeled DSBP were abundant proteins in the 40 to 200 kDa range (Fig 6A). Most DSBP in this molecular weight range did not vary significantly with low level H$_2$O$_2$ exposure, although IMPDH-II (arrow) displayed increased disulfide bonding at higher oxidant levels (Fig. 6A). In contrast, a number of spots in the 15 to 30 kDa range (Fig 6B) were found that were not readily detectable by silver staining (Fig 2D). As expected, peroxiredoxins 1 and 2 (arrow head) displayed a reduction in disulfide bonding with increasing levels of H$_2$O$_2$ exposure (Fig 6B). Higher concentrations of H$_2$O$_2$ also resulted in increased disulfide bonding of several proteins (asterisk) in the 30 kDa range (Fig 6B). Interestingly, some DSBP appeared unique to one level of H$_2$O$_2$ exposure.
exposure and not to other levels, suggesting that disulfide bonding in the cytosol is affected in a H$_2$O$_2$ concentration-dependent manner.

**Epitope-tagged Hsp70 forms mixed-disulfides with β4-spectrin and APC**

Although the disulfide-linked proteins we analyzed by immunoblotting appeared as higher molecular weight species under non-reducing conditions (Fig. 4), we could not determine if the protein complexes were composed of either homo- or heterodimeric species linked by disulfide bonds. Mixed disulfide bond formation is known to occur between nascent polypeptides and oxidoreductases in the ER and between glutathione and redox sensitive proteins in the cytoplasm (4, 15, 16, 42). To determine if the high molecular weight species observed under nonreducing conditions were mixed-disulfides we expressed an epitope-tagged version of Hsp70 (Hsp70-Flag) in HT22 cells and assayed disulfide-linked complexes by both immunoprecipitation and immunoblotting with a Flag-specific antibody (Fig. 7). Similar to endogenous Hsp70 (Fig. 4C), Hsp70-Flag appeared as several high molecular weight species under nonreducing conditions (Fig. 7A). These disulfide-linked species disappeared after the addition of reducing agents (Fig. 7A). In order to isolate and identify the disulfide-linked protein partners of Hsp70-Flag, we performed Redox 2D-PAGE analysis of the precipitated immunocomplexes. Following silver-staining, the reduced monomeric form of Hsp70 was observed as an intense 70 kDa spot on the diagonal line (Fig. 7C). However, intramolecular or intermolecular disulfide-linked forms of Hsp70 appeared on either the left or right side of the diagonal line respectively. The Hsp70 spot that appeared to the extreme right of the diagonal correlated with an approximately 260 kDa spot immediately above it (Fig. 7C). We determined that
the 260 kDa spot was composed of both β4-Spectrin (Q8VIE5) and Adenomatous polyposis coli (APC) protein (Q61315) following MALDI-TOF and MS/MS analysis. Therefore, the identity of heterodimeric pairing proteins linked by a disulfide bond can be determined by immunoprecipitating the bait protein with an epitope tag under non-reducing conditions, resolving the immunocomplex by Redox 2D-PAGE and finally identifying the disulfide-linked protein(s) by mass spectrometry. Furthermore, by comparing both coordinate axes in Redox 2D-PAGE, one can determine the nature of the disulfide pairing (i.e. hetero- or homodimeric disulfide bonding).
Discussion

In this study, we identified several proteins known to form disulfide bonds during part of their catalytic cycle, including peroxiredoxins, NDPK, ribonucleotide reductase and thioredoxin reductase. The identification and the change in disulfide bonding properties of these proteins following oxidant exposure, confirms the validity of the Redox 2D-PAGE technique. Although the cytosol has a reducing environment that should prohibit stable disulfide bond formation (39), we observed numerous DSBP previously not known to undergo disulfide bond formation under both non-stressed and pro-oxidant conditions (Fig. 2). The formation of DSBP in non-stressed cells is likely because reactive Cys-SH groups of redox sensitive proteins can exist as thiolate anions at physiological pH and can readily form mixed disulfide bonds with glutathione (glutathionylation) or redox sensitive proteins (4, 6).

We observed that H$_2$O$_2$ and diamide treatment strongly promoted both glutathionylation (Fig. 1C) and protein disulfide bond formation (Fig. 2D,E). Glutathionylation has been implicated in buffering of oxidative stress, regulation of enzyme activity and protection against potentially irreversible protein oxidation or polymerization (4, 42,43). For example, H$_2$O$_2$- or diamide-treated thioredoxin is converted into an enzymatically inactive homodimer via a Cys-72-Cys-72 intermolecular disulfide bond (44). Glutathionylation of the Cys-72 residue of thioredoxin prevents dimerization and is reversible (45). Therefore, cysteine residues of proteins that are readily glutathionylated may also be susceptible to either intra or intermolecular disulfide bonding. Indeed, we observed a significant overlap with the DSBP we detected in our study, and the glutathionylated proteins
found in separate studies, including Hsc70, Hsp70, Hsp90, tcp-1, EF-1, enolase, GAPDH, lactate dehydrogenase, pyruvate kinase, nucleoside diphosphate kinase, tubulin β, peroxiredoxin 1 and IMPDH-II (46-49). However, during exposure to high levels of ROS or during a drop in the GSH/GSSG ratio, glutathionylation may give way to protein disulfide bond formation. In this study, treatment of cytosolic extracts with increasing levels of GSSG promoted disulfide bonding within a select group of proteins (Fig 5), some of which were also observed to undergo disulfide bonding following H₂O₂ or diamide treatment (Fig 2D,E). It is interesting to note that glutamate treatment induced a large drop in total cellular GSH but had little effect on Prot-SSG levels (Fig. 1C) and produced only a modest increase in DSBP levels (Fig. 2C). Exposure to high levels of H₂O₂ and diamide may promote disulfide bonding at least partly by bringing about alterations in the GSH:GSSG equilibrium, ultimately leading to large increases in glutathionylation. Thus, glutathionylation may serve as a mechanism that protects redox sensitive proteins from potentially irreversible disulfide bond formation during exposure to low levels of oxidants but may facilitate protein disulfide bonding during severe oxidative stress.

A number of the DSBPs identified in our analysis were either involved in protein translation or protein folding (Table 1). The DSBPs involved in translation include elongation factor-1-γ (EF-1-γ), elongation factor 2 (EF-2), cysteinyl-tRNA synthetase (CysRS) and histidyl-tRNA synthetase (HisRS). Proteins involved in protein folding, such as heat shock protein 90 (Hsp90), heat shock cognate protein 70 (Hsc70) and several t-complex polypeptide-1 (tcp-1) isoforms, also exhibited increased disulfide bonding after diamide and H₂O₂ exposure (Fig. 2). Several
reports have examined the connection between protein oxidation, translational fidelity and bacterial cellular senescence (50). In studies that used a carbonylation detection assay to measure protein oxidation, Hsp70 and a number of translation elongation factors were increasingly oxidized in growth-arrested *Escherichia coli* cells (50). In a recent study, several yeast translation factors were shown to undergo glutathionylation with a concomitant inhibition of protein synthesis following H$_2$O$_2$ exposure (49). Furthermore, in a yeast mutant strain lacking glutathione, protein synthesis was irreversibly inhibited by H$_2$O$_2$ exposure (49). This leads to the conclusion that translational efficiency and fidelity, in addition to protein folding, may be compromised in aged or oxidant-stressed cells (50). The results presented here lend support to this conclusion.

H$_2$O$_2$ has been used extensively to examine redox regulation of several physiological processes including signal transduction, response to oxidative stress, cell proliferation and apoptosis (51). Most studies supporting a regulatory role for H$_2$O$_2$ use high exogenous concentrations due to its rapid removal by intracellular catalase and peroxidases. In fact, the gradient between extracellular and cytosolic concentrations of H$_2$O$_2$ is estimated to be about seven fold (52). While the concentration of H$_2$O$_2$ used in our initial experiments was high (1-10 mM), and comparable levels can be produced during the acute oxidative burst in neutrophils during phagocytosis of bacteria (40), these levels are generally not representative of normal physiological conditions. We therefore examined disulfide bond formation in HT22 cells after exposure to concentrations of H$_2$O$_2$ (10-400 µM) that are non-toxic and are considered to be in the range found *in vivo* (41). A number of studies have shown that extremely low concentrations of H$_2$O$_2$ (3-15 µM) can
stimulate cell growth, low concentrations (120-150 µM) can induce a transient adaptive response, and intermediate concentrations (250-400 µM) can cause permanent loss of replicative or divisional competence (51). In our study we saw a H$_2$O$_2$ concentration-dependent increase in disulfide bonding of inosine-5'-monophosphate dehydrogenase 2 (Fig 6A) an essential enzyme involved in the generation of precursors for DNA synthesis. At high levels of H$_2$O$_2$ (10 mM), which can be generated by phagocytic cells (40), we also observed an increase in disulfide bonding of ribonucleotide reductase and nucleoside diphosphate kinase B (Fig 2D), two enzymes also essential for DNA synthesis. The formation of disulfide bonds within these proteins following exposure to high levels oxidants may inhibit their activity, decrease DNA synthesis and contribute to H$_2$O$_2$-mediated growth inhibition.

In contrast to intermediate and high level H$_2$O$_2$ exposure, low level exposure of H$_2$O$_2$ (10 µM) had little effect on the disulfide bonding of proteins in the 40 to 150 kDa range (Fig 6A). However, several lower molecular weight disulfide-linked proteins (Fig 6B) were seen in cells treated with 10 µM H$_2$O$_2$ but were not observed in cells treated with higher levels of H$_2$O$_2$ (150-400 µM). A number of DSBP also appeared to be unique to either 150µM or 400µM H$_2$O$_2$ treatment (Fig 6B). Therefore, the proteins that appeared to be uniquely disulfide bonded at low levels of H$_2$O$_2$ exposure may be involved in the mitogenic effects of low oxidant concentrations on cultured cells (51). In contrast, proteins that are disulfide-linked at intermediate H$_2$O$_2$ concentrations, may participate in the adaptive response or growth arrest observed in cells cultured with intermediate oxidant concentrations
Due to their low abundance we have been unable to isolate and identify these low molecular weight proteins.

Although molecular chaperones such as calnexin, calreticulin and BiP are known to assist protein folding and disulfide bond formation in the ER (16), few examples of mixed disulfides formed between chaperones and other proteins exist. A recent study demonstrated that a plant thioredoxin related protein, AtTDX, interacts via its oxidant-sensitive thioredoxin domain with Ssb2, a yeast orthologue of mammalian Hsp70 (53). However, it is unknown if AtTDX and Ssb2 form a mixed disulfide. The data presented here shows that Hsp70 forms mixed disulfides with other cytoplasmic proteins (\(\beta_4\)-spectrin and APC). Whether this interaction affects the function or folding of these proteins awaits further investigation.

Numerous studies have shown a clear link between protein oxidation and both aging and neurodegenerative diseases (2, 4, 54-56). However, the exact mechanism by which protein oxidation affects enzymatic activity and signal transduction processes during normal and pathological conditions is poorly understood. Moreover, the role of inappropriate disulfide bond formation in the pathogenesis of human disease is relatively unknown. The physiological importance of maintaining key redox sensitive proteins in a reduced state was recently demonstrated in studies showing that the Fanconi anemia group C protein increases the survival of hematopoietic progenitor cells by preventing disulfide bond formation and the concomitant inactivation of glutathione-S-transferase P1 during apoptosis (30) in addition to preventing \(\text{H}_2\text{O}_2\)-induced activation of the redox sensitive kinase ASK1 (57). The findings presented here highlight the pivotal role
of cysteine oxidation and disulfide bond formation in many aspects of cell function during oxidative stress and should provide a basis for further exploration of the disulfide proteome.

Acknowledgements

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References


Figure Legends

Figure 1. Measurement of GSH (A), GSSG (B) and Prot-SSG (C) levels during oxidative conditions. Intracellular glutathione levels were determined as described in “Materials and Methods”. Cells were either untreated (control) or treated with 5 mM glutamate for 8 hours, 10 mM H$_2$O$_2$ for 5 minutes or 1 mM diamide for 5 minutes. Data represent the average of two separate experiments.

Figure 2. Separation of cytosolic DSBP by Redox 2D-PAGE. Oxidant treated HT22 Cells were separated into cytosol, membrane/organelle and nuclear fractions using differential detergent fractionation. To ensure that the cytoplasmic fractions were not contaminated with proteins from either the membrane/organelle or nuclear fractions, immunoblot analysis of the fractions was performed with antibodies that recognize an endoplasmic reticulum (PDI) and nuclear specific protein (CREB) (A). Essentially no contamination with proteins from the endoplasmic reticulum or the nucleus was observed in the cytoplasmic fraction. As a loading control, blots were probed with an anti-actin antibody. Control (C), Glutamate-treated (G), H$_2$O$_2$-treated (H), diamide-treated (D) cells and whole cell extract (WCE). Cytosolic protein extracts from HT22 cells were sequentially resolved by nonreducing and reducing SDS-PAGE followed by silver staining. Cells were either untreated (B), or treated with 5 mM glutamate for 8 hours (C), 10 mM H$_2$O$_2$ for 5 minutes (D) or 1 mM diamide for 5 minutes (E). As a negative control, the reducing agent dithiothreitol (100mM) was added in both dimensions to reveal a prominent diagonal line with no spots in the off-diagonal zone (F). The gels presented are representative of three separate analyses.
Figure 3. Representative Redox 2D-PAGE gel of all DSBP. Cytosolic extracts from cells treated with 1 mM diamide for 5 minutes were resolved by Redox 2D-PAGE, silver stained and numbered accordingly (A). Corresponding protein spot identities are shown in Table 1. Magnification of DSBP in the above gel revealing proteins in the 40 to 100 kDa range (B).

Figure 4. Analysis of DSBP by non-reducing SDS-PAGE and immunoblotting. Cytoplasmic extracts from HT22 cells treated with either 5 mM Glutamate, 10 mM diamide or 1 mM H₂O₂ were resolved by nonreducing SDS-PAGE (4-12% gel), electroblotted on PVDF membrane and hybridized with antibodies against peroxiredoxin 1 (A), nucleoside diphosphate kinase B (B), heat shock protein 70 (C), heat shock cognate protein 70 (D), glyceraldehyde-3-phosphate dehydrogenase (E), and α-tubulin (F). As a control, lysates were reduced using 100 mM DTT (left side lanes). Proteins that form disulfide bonds exhibit slower migration and thus appear as higher molecular weight bands. The reduced monomeric form of the protein is indicated by an arrow.

Figure 5. Decreasing the GSH:GSSG ratio promotes disulfide bonding in cytoplasmic proteins. Cytoplasmic extracts (250 µg) were mixed with either 5mM GSH alone (A) or different ratios of reduced and oxidized glutathione expressed as 10:1 (B), 1:1 (C) and 1:10 (D). The concentration of glutathione equivalents was maintained at 5 mM. Proteins were analyzed by Redox 2D-PAGE and silver staining. Decreasing the GSH:GSSG ratio promoted disulfide bonding of specific
proteins (indicated by arrows) including calreticulin (1), Hsp70 (2), Tcp-1β (3), Bcd orf2 (4), GAPDH (5) and peroxiredoxin 1 (6).

**Figure 6. Low levels of H$_2$O$_2$ promote disulfide bonding in a concentration-dependent manner.** HT22 cells were exposed to several concentrations of H$_2$O$_2$ (10-400 µM) for 10 minutes and cytoplasmic protein extracts were separated by Redox 2D-PAGE followed by labeling of DSBP with biotin-conjugated iodoacetamide. After electroblotting, labeled proteins were detected with streptavidin-conjugated HRP and ECL. Blots of proteins in the 40-150 kDa range (A) were exposed for 1 minute. Inosine monophosphate-dehydrogenase II (indicated by arrows) displayed increased disulfide bonding with higher H$_2$O$_2$ concentrations. Blots of separated proteins in the 15 to 35 kDa range (B) were exposed for a longer time (5 minutes) and revealed decreased disulfide bonding of peroxiredoxin 1 (indicated by arrow heads) with higher H$_2$O$_2$ concentrations (150-400mM). Some lower molecular weight DSBP displayed a concentration-dependent increase in disulfide bond formation (indicated by asterisk). Other low molecular weight DSBP appeared unique to each concentration of H$_2$O$_2$.

**Figure 7. Epitope-tagged Hsp70 dimerizes with β4-Spectrin and APC via an intermolecular disulfide bond.** FLAG-tagged Hsp70 or the empty vector (pRev) were expressed in HT22 cells and cytosolic extracts were immunoprecipitated using agarose-coupled anti-FLAG resin. Immunoblotting of the resulting samples with anti-FLAG antibody revealed several high molecular weight complexes, including an approximately 260 kDa species (indicated by arrow) under
nonreducing conditions (A). Treatment of cells with 5 mM H$_2$O$_2$ for 5 minutes resulted in the increased detection of several disulfide-linked Hsp70 complexes in cytosolic extracts but only the 260 kDa species in the immunoprecipitated fraction. The addition of 150 mM $\beta$-mercaptoethanol to the samples resulted in the sole appearance of the reduced monomeric form of Hsp70. Immunoprecipitated fractions from pRev (B) and Hsp70 (C) expressing cells were resolved by Redox 2D-PAGE and silver stained. The reduced monomeric form of Hsp70 appeared as an intense 70 kDa spot on the diagonal line (indicated by arrow head). An intramolecular disulfide-linked form of Hsp70 appeared on the left side of the diagonal (indicated by asterisk). The intermolecular disulfide-linked Hsp70 spot that appeared to the extreme right of the diagonal correlated with a 260 kDa spot immediately above it (indicated by arrows). The 260 kDa spot was identified as a mixture of $\beta$4-spectrin and Adenomatous polyposis coli (APC) protein by both MALDI-TOF and MS/MS. The identity of both $\beta$4-spectrin and APC was confirmed in two separate experiments
Table I: Summary of disulfide-bonded proteins (DSBP) identified and method of identification

Cytosolic DSBP were isolated by Redox 2D-PAGE, in-gel digested with trypsin and identified by either matrix-assisted laser-desorption ionization time of flight (MALDI-TOF) or liquid chromatography-electrospray tandem mass spectrometry (MS/MS)

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Fig. 1 Cumming et al.

A

GSH

nmol/mg protein

B

GSSG

Control
Glutamate
H2O2
Diamide

nmol/mg protein

C

Prot-SSG

nmol/mg protein
Fig. 7. Cumming et al.
Protein disulfide bond formation in the cytoplasm during oxidative stress
Robert C. Cumming, Nancy L. Andon, Paul A. Haynes, Minkyu Park, Wolfgang H. Fischer and David Schubert

J. Biol. Chem. published online March 18, 2004

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