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Bifunctional Electrophiles Cross-Link Thioredoxins with Redox Relay Partners in Cells

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Abstract

Thioredoxin protects cells against oxidative damage by reducing disulfide bonds in improperly oxidized proteins. Previously, we found that the baker's yeast cytosolic thioredoxin Trx2 undergoes cross-linking to form several protein-protein complexes in cells treated with the bifunctional electrophile divinyl sulfone (DVSF). Here, we report that the peroxiredoxin Tsa1 and the thioredoxin reductase Trr1, both of which function in a redox relay network with thioredoxin, become cross-linked in complexes with Trx2 upon DVSF treatment. Treatment of yeast with other bifunctional electrophiles, including diethyl acetylenedicarboxylate (DAD), mechlorethamine (HN2), and 1,2,3,4-diepoxybutane (DEB), resulted in the formation of similar cross-linked complexes. Cross-linking of Trx2 and Tsa1 to other proteins by DVSF and DAD is dependent on modification of the active site Cys residues within these proteins. In addition, the human cytosolic thioredoxin, cytosolic thioredoxin reductase, and peroxiredoxin 2 form cross-linked complexes to other proteins in the presence of DVSF, although each protein shows different susceptibilities to modification by DAD, HN2, and DEB. Taken together, our results indicate that bifunctional electrophiles potentially disrupt redox homeostasis in yeast and human cells by forming cross-linked complexes between thioredoxins and their redox partners.

Introduction

Organic electrophiles constitute an important class of toxins that may be taken up by cells from their environments or produced during intracellular processes.[1,2] These molecules react with a number of intracellular nucleophiles, including the small molecule detoxicant glutathione, nitrogenous bases in DNA, and nucleophilic amino acids in proteins, thereby exhibiting a variety of dose-dependent biological activities.[3,4] Cells exposed to low concentrations of electrophiles often activate cytoprotective gene expression in cells, whereas higher concentrations of these molecules can lead to cell cycle arrest and cell death.[3,5-7] In particular, many electrophiles react readily with Cys residues in target proteins, largely due to the pronounced nucleophilicity of thiol groups among biological

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Supporting Information Available.
Supplementary methods, PCR primer sequences, summary of proteomic data, toxicity data, representative mass spectra, supplementary protein cross-linking data, and genotypic and phenotypic characterization of yeast strains. This information is available free of charge via the Internet at http://pubs.acs.org/.

nucleophiles.[8-10] Target proteins that are susceptible to electrophilic modification often contain hyper-reactive Cys residues, which usually exist in their deprotonated thiolate state due to the local protein environment.[11,12]

Many of the protein targets of electrophiles use reactive Cys residues to fulfill their normal biological function.[13,14] For example, the redox active Cys residues in thioredoxin (Trx) proteins are necessary to reduce disulfide bonds in proteins undergoing oxidation.[15,16] Although the substrate profile of Trx is quite diverse, many Trx substrates provide direct defense against oxidants generated during stress. This is true for the peroxiredoxins (Prdxs), a family of thiol-dependent peroxidases that undergo intermolecular disulfide formation upon oxidation.[17-20] During the thioredoxin catalytic cycle, a reduced Trx carries out a disulfide exchange reaction with an oxidized substrate (e.g., an oxidized Prdx) to restore it to its native, functional state. Oxidized Trx is subsequently reduced by Trx reductase (TrxR), a disulfide reductase that uses NADPH as a reducing agent.[15] Due to the presence of nucleophilic residues in their active sites, Trx and TrxR are considered high affinity targets for electrophilic modification and inactivation,[13,14] and electrophilic inhibitors of this redox relay are emerging as potential anticancer agents.[21,22]

A number of structurally unrelated electrophiles modify Trx and TrxR proteins isolated from different organisms. Several of these molecules contain multiple electrophilic centers, including the lipid peroxidation products 4-hydroxyonenal and acrolein,[23,24] the nitrogen mustard cyclophosphamide,[25] and the natural product curcumin.[26,27] Previously, we determined that two bifunctional electrophiles–divinyl sulfone (DVSF) and diethyl acetylenedicarboxylate (DAD)–cross-link redox active proteins, including recombinant Trx2 from *Saccharomyces cerevisiae*, in vitro.[28,29] Moreover, these molecules cause the formation of cross-links between Trx2 and other proteins in yeast.[29] Here, we establish the identity of the proteins that form cross-links with Trx2 in cells as the peroxiredoxin Tsa1 and the thioredoxin reductase Trr1. Each of these proteins is targeted by structurally diverse bifunctional electrophiles and undergoes cross-linking to other proteins in both *S. cerevisiae* and mammalian cells. Collectively, our results suggest a potential mechanism through which industrial and medically relevant bifunctional electrophiles may perturb redox homeostasis in cells and highlight the utility of bifunctional electrophiles in stabilizing interactions between redox relay partners.

Experimental Procedures

Chemicals

DVSF, DAD, 1,2,3,4-diepoxybutane (DEB), mechlorethamine hydrochloride (nitrogen mustard, HN2), EVSF, DEM, and *N*-ethylmaleimide (NEM) were purchased from Sigma (Fig. 1, St. Louis, MO). All molecules were dissolved in Me_2SO, with the exception of HN2, which was dissolved water, and NEM, which was dissolved in EtOH.

Yeast and Mammalian Cell Culture

The strains of *S. cerevisiae* used in this study were obtained from Open Biosystems (Huntsville, AL) and include BY4741 (wild-type strain; MAT**a** his3Δ1 leu2Δ0 met15Δ0 ura3Δ0), Trx2-TAP, and Tsa1-TAP. A *tsa1Δ tsa2Δ* mutant strain (MAT**a** his3Δ1 leu2Δ0 met15Δ0 ura3Δ0; *tsa1*^{R}; *tsa2::HIS3*) was created as described in the Supplementary Materials and Methods. All strains were maintained in YPD medium at 30°C; when transformed with expression plasmids, cells were maintained in uracil dropout medium (MP Biomedicals, Solon, OH). RKO human colorectal carcinoma cells were obtained from ATCC and were a kind gift of Lawrence J. Marnett (Vanderbilt University School of Medicine). RKO cells were maintained in DMEM supplemented with 10% fetal bovine

serum (Hyclone, Rockford, IL), 2 mM L-glutamine (Invitrogen, Grand Island, NY), and antibiotic-antimycotic (Invitrogen) in a humidified atmosphere at 37°C in 5% CO_2.

Cloning and Site-Directed Mutagenesis

Genes encoding FLAG-tagged Trx2, Tsa1, and Trr1 were amplified using standard PCR procedures and the primer sets shown in Supplementary Table 1. After amplification, the FLAG-tagged genes were cloned into the *S. cerevisiae* expression vector p416-GPD via *Spe*I and *Xho*I sites. For recombinant Tsa1 production in *E. coli*, the Tsa1 coding sequence was amplified from *S. cerevisiae* genomic DNA with the primer sequences indicated in Supplementary Table 1 and cloned into pET45b (Novagen) via *Kpn*I and *Xho*I sites. Mutation of the codons encoding Cys in Trx2 and Tsa1 was accomplished using the QuikChange protocol (Agilent, Santa Clara, CA) with the primer sequences indicated in Supplementary Table 1. All cloned genes and site-directed mutants were verified by DNA sequencing.

Immunoblotting

Protein lysates were prepared from yeast and mammalian cells as previously described.[28,29] Lysates were quantified using either the Bradford assay (Sigma) or bicinchoninic acid assay (ThermoScientific, Rockford, IL) with bovine serum albumin as a standard. Equal amounts of proteins (usually between 10-40 μg) were diluted in 3X Laemmli sample buffer (60 mM Tris, pH 6.8; 0.7 M 2-mercaptoethanol; 2% (w/v) SDS; 10% (v/v) glycerol; 0.33 mg/mL bromophenol blue)), resolved using SDS-PAGE, and transferred to PVDF membranes for 2 h at 50 V. Membranes were blocked in TBS-T (100 mM Tris (pH 7.5), 150 mM NaCl, and 0.1% (v/v) Tween 20) containing 5% (w/v) non-fat dry milk. Primary antibodies recognizing the FLAG epitope (mouse monoclonals, Agilent Technologies or Sigma), protein A (rabbit polyclonal, Sigma), Pgk1 (mouse monoclonal, Invitrogen), TrxR1 (rabbit polyclonal, Epitomics (Burlingame, CA)), Trx1 (mouse monoclonal, BD Pharmingen (San Jose, CA)), Prdx2 (rabbit polyclonal, Sigma), or α-tubulin (rabbit polyclonal, Cell Signaling (Beverly, MA)) were diluted in blocking buffer and incubated with membranes for 2 h at room temperature or overnight at 4°C. Subsequently, membranes were washed three times with TBS-T for 5 min, incubated with secondary antibodies conjugated to horseradish peroxidase (Cell Signaling Technology) for 45 min at room temperature, and washed four times with TBS-T for 15 min. Proteins were visualized using enhanced chemiluminescent detection.

Immunoprecipitations

Immunoprecipitations were conducted on 100 μg-2 mg of protein lysates from Me_2SO or DVSF-treated yeast cells diluted to 700 μL in yeast lysis buffer (20 mM Tris (pH 8), 0.5 mM EDTA, 10% glycerol, 50 mM NaCl, and a protease inhibitor cocktail). EZView anti-FLAG affinity gel (10-20 μL; Sigma) was added to each reaction, and samples were rotated at 4°C for 4 h. Beads were pelleted for 2 min at 4°C, 11,000 × *g* and washed eight times with lysis buffer containing 0.1% (w/v) SDS and 0.5% (w/v) sodium deoxycholate. Proteins were eluted from beads with 15-30 μL elution buffer (50 mM Tris (pH 7.5), 150 mM NaCl, 0.5 mg/mL 3X FLAG peptide (Sigma)) for 30 min at room temperature. Beads were pelleted at 11,000 × *g* for 2 min, and the supernatant was recovered, boiled in Laemmli sample buffer, and resolved on SDS-PAGE. Proteins were detected with Coomassie blue staining or immunoblot with an antibody against the FLAG tag.

Identification of Proteins Cross-Linked to FLAG-Trx2

Following immunoprecipitation of Trx2, peptides were isolated from 14 equally sized gel slices spanning a molecular weight range of approximately 10-100 kDa. Gel slices were subject to repeated dehydration/rehydration steps in 2:1 acetonitrile:25 mM NH_4HCO_3,

reduction with DTT, alkylation with iodoacetamide, and further dehydration/rehydration steps as described elsewhere.[30] Dried gel slices were rehydrated with approximately 10 μL of 12.5 ng/μL sequencing-grade trypsin (Promega, Madison, WI), to which 50 μL 25 mM NH_4HCO_3 was added, prior to incubation overnight at 37°C. Peptides recovered from in-gel trypsin digests were resolved and analyzed using an Agilent 1100 HPLC System (Santa Clara, CA) in line with an electrospray linear ion trap mass spectrometer (LTQ-XL, Thermo Scientific, West Palm Beach, FL). Peptides were trapped and desalted on an Agilent Eclipse XDB-C8 2.1 mm × 15 mm trap (Santa Clara, CA) with mobile phases A (0.1% formic acid in water) and B (0.1% formic acid in acetonitrile). Eluted peptides were subsequently resolved on a Vydac Everest reverse-phase C18 monomeric column (2.1 mm × 150 mm, 300 Å, 5 μm; Albany, OR) with a gradient of 5% to 60% B in 60 min at a flow rate of 200 μL/min. Peptide and protein identification were performed using Mascot (Matrix Science, Boston, MA). Spectra derived from fragmented peptides were analyzed using Mascot MS/MS Ion searches against the SwissProt database for the *S. cerevisiae* proteome. Carbamidomethylation and methionine oxidation were permitted in the database searches, with variations in the peptide and fragment masses being tolerated up to 1.2 and 0.6 Da, respectively.

Cytotoxicity Assays

The toxicity of BDE and HN2 in yeast and mammalian cells was measured using procedures described elsewhere.[28,29]

Expression of Recombinant Tsa1

The expression plasmid pET45b-Tsa1 was transformed into BL21(DE3) cells. Liquid cultures (250 mL) were grown to mid-log phase and induced for 3 h with 1 mM isopropyl β-D-1-thiogalactopyranoside at 37°C. Recombinant Tsa1 was purified from the cells using NiNTA Fast Start His Tag purification protocol (Qiagen, Valencia, CA) under native conditions. Following elution, imidazole was removed by buffer exchange into protein storage buffer (50 mM Tris (pH 7.5), 2 mM DTT, 10% glycerol, and protease inhibitor cocktail (ThermoFisher, Rockford, IL)) using a PD Minitrap G25 Sephadex column (GE Healthcare, Piscataway, NJ). Prior to biochemical assays, 30-50 μL wild-type or mutant Tsa1 at ~200 μM were thawed and reduced with 20 mM tris(2-carboxyethyl)phospine for 30 min at 37°C. Excess reductant and storage buffer were removed by centrifuging samples through a Micro Bio-Spin 6 gel filtration column (BioRad, Hercules, CA) equilibrated with 50 mM Tris (pH 7.0) for 1 min at 1000 × *g*. Recovered Tsa1 was quantified using an estimated molar absorptivity of 23,950 $M^{-1}•cm^{-1}$.

Treatment of Recombinant Tsa1 with Protein Cross-Linkers

Reactions containing 50 mM Tris (pH 7.0), 2.5 mM EDTA, 150 mM NaCl, 10 μM reduced Tsa1, and Me_2SO or 100 μM electrophiles dissolved in Me_2SO were incubated for 24 h at 37°C. Reactions were terminated with the addition of 10 μL 3X Laemmli sample buffer supplemented with 30 mM DTT. Samples were boiled at 95°C for 5 min and resolved using SDS-PAGE. Gels were stained with Coomassie brilliant blue to visualize proteins.

Monitoring Oxidation of Tsa1 Following Treatment with *N*-Ethylmaleimide

Reactions (60 μL) containing 50 mM Tris (pH 7.0), 2.5 mM EDTA, 150 mM NaCl, 10 μM reduced Tsa1, and varying concentrations of NEM or EtOH (solvent) were incubated for 3 h at 37°C. Following incubation, reactions were centrifuged through a Micro Bio-Spin 6 gel filtration column (BioRad) equilibrated with 50 mM Tris (pH 7.0) for 1 min to remove unreacted NEM. Samples were split into 18 μL aliquots and treated for 15 min with 100 μM H_2O_2 or H_2O as a control, followed by the addition of 3X non-reducing Laemmli sample

buffer with or without 30 mM DTT. Samples were heated for 5 min at 95°C and resolved using SDS-PAGE. Protein complexes were visualized by staining gels with Coomassie brilliant blue.

Results

Tsa1 and Trr1 Form Covalent Complexes with Trx2 in DVSF-Treated Yeast Cells

Recently, we found that TAP-tagged Trx2 becomes cross-linked to other proteins in cells treated with sub-cytotoxic and toxic concentrations of DVSF.[29] Likewise, multiple cross-linked species containing FLAG-Trx2 were observed in lysates of cells treated with 300 μM DVSF, a dose where limited acute toxicity is observed (Fig. 2A and B).[29] To identify the proteins cross-linked to Trx2, FLAG-Trx2 was immunoprecipitated from lysates of yeast cultures following exposure to Me_2SO or DVSF and resolved on SDS-PAGE (Fig. 2C). Tryptic peptides derived from immunoprecipitated proteins were subsequently sequenced using mass spectrometry. Upon analysis of the mass spectra (Supp. Figs. 1-3), proteins found in the isolated complexes were Trx2 (or Trx1), Trr1 (cytosolic Trx reductase 1), and Tsa1 (a cytosolic 2-Cys peroxiredoxin). Trr1 and Tsa1 were not present at observable levels in Trx2 immunoprecipitated from cells treated with Me_2SO (i.e., vehicle), suggesting that both form covalent cross-links to Trx2 in the presence of DVSF. In contrast, several proteins (e.g., the ADP ribosylation factor Arf1, the translation elongation factor Tef1, and the molecular chaperones Ssa1 and Hsc82, Supp. Table 2) immunoprecipitated with Trx2 in the absence or presence of DVSF and, thus, were not included in further analyses.

To validate that Trx2 forms cross-links with Tsa1, we expressed FLAG-Tsa1 in the Trx2-TAP strain and immunoprecipitated the FLAG-tagged proteins following Me_2SO or DVSF treatment. Trx2-TAP was observed in at least two cross-linked complexes with Tsa1-FLAG, matching up closely with the molecular weight of Tsa1-FLAG complexes (Fig. 3A). Of the two main complexes observed, one of these likely corresponds to a monomer of Tsa1 cross-linked to a single molecule of Trx2 (at ~56 kDa), whereas the other complex may reflect a single molecule of Trx2 covalently bound to a cross-linked dimer of Tsa1 (at ~78 kDa). Using a genetic approach, we found that many of the species cross-linked to FLAG-Trx2 were not observed in yeast cells lacking the *TSA1* and *TSA2* genes (*tsa1Δ tsa2Δ*, Figure 3B), suggesting that multiple covalent complexes form between Trx2 and Tsa1 in the presence of DVSF. Immunoprecipitation of FLAG-Trr1 did not show pronounced cross-linking between it and Trx2-TAP in response to DVSF treatment (data not shown), which may be due to less cross-linking occurring with the FLAG-Trr1 protein in general. However, treatment of the Trr1-TAP strain with DVSF revealed a cross-linked species approximately 12 kDa higher than the Trr1-TAP monomer (Supp. Fig. 4). The weight of this complex is consistent with the formation of a Trr1-Trx2 cross-link. Taken together, these results indicate that Trx2 forms several stable cross-linked complexes with known redox partners–Tsa1 and Trr1–in cells treated with DVSF.

Structurally Unrelated Bifunctional Electrophiles Modify Trx2, Tsa1, and Trr1

We next sought to determine whether other bifunctional electrophiles form covalent cross-links between Trx2, Tsa1, and Trr1 similar to those formed with DVSF. Notably, we tested whether the bifunctional electrophiles DAD (an industrial chemical), DEB (a toxic metabolite of butadiene and a proposed product of the prodrug treosulfan),[31,32] and HN2 (a cancer chemotherapeutic)[33] also form cross-linked complexes with these proteins (Fig. 1). Initially, we determined the toxicity of DEB and HN2 in yeast in order to establish appropriate doses of these molecules for further studies (Supp. Fig. 5A). At sub-cytotoxic doses of DEB and cytotoxic doses of both DAD and HN2,[29] we observed that each of these bifunctional electrophiles promotes cross-linking of Trx2 and Tsa1 in yeast to form

complexes of similar molecular weights to those formed with DVSF (Fig. 4A & B). Cross-linking of FLAG-Trr1 to other cellular proteins was less pronounced for these electrophiles, although at least three distinct cross-linked complexes were observed in cells treated with DVSF, DAD, and HN2 (Fig. 4C). Less cross-linking of proteins to FLAG-Trx2 was observed for all cross-linkers in the *tsa1Δ tsa2Δ* strain, implying that each of these molecules can cross-link Trx2 and Tsa1 to form similar complexes (Supp. Fig. 6). Collectively, these results suggest that Trx relay proteins within a cell are subject to similar modification by structurally diverse bifunctional electrophiles.

Trx2 and Tsa1 Modification by Bifunctional Electrophiles Is Mediated by Modification of Active Site Cys Residues

Trx2, Tsa1, and Trr1 use redox active Cys residues to carry out specialized redox reactions in the cell. To determine whether the active site Cys residues in Trx2 and Tsa1 undergo modification and thereby promote cross-linking, we exposed yeast expressing wild-type or mutant forms of these enzymes to DVSF or DAD and monitored molecular weight changes in these proteins. In cells expressing Trx2 with a mutation of the catalytic Cys to Ala (C31A), there was a considerable decrease in the number of cross-links formed following treatment with DAD or DVSF, with a single complex at ~55 kDa being the most notable complex formed under both conditions (Fig. 5A). However, when the resolving Cys was mutated (C34A), cross-linking of other proteins to Trx2 by DVSF was still observed, albeit to a lesser extent than with the wild-type protein. These results suggest that the majority of cross-links formed with binding partners are due to modification of the catalytic Cys within Trx2. Mutation of either the peroxidatic Cys (C47) or resolving Cys (C170) in Tsa1 to Ala resulted in a distinct decrease in cross-links formed in cells treated with DAD or DVSF, as did mutation of both Cys residues to Ala (Fig. 5B). Similar results were obtained when recombinant Tsa1 proteins containing these mutations were treated with DAD and DVSF (Supp. Fig. 7), which principally formed cross-linked dimers. Taken together, these results indicate that the active site Cys residues in both Trx2 and Tsa1 are modified by bifunctional electrophiles to mediate protein cross-linking. In addition, they suggest that DVSF and DAD generally modify target proteins on Cys residues in a cellular context, rather than targeting other nucleophilic residues in these proteins.

Since the active site Cys residues within thioredoxin relay proteins are targets of bifunctional electrophiles, alkylation should inactivate these proteins. Consistent with this idea, a wide range of electrophiles inhibit Trx and TrxR proteins from different species.[16,22] However, there is some discrepancy about the extent to which peroxiredoxins are modified and inhibited by electrophiles.[34] Therefore, we determined whether Tsa1 can undergo oxidation following modification by *N*-ethylmaleimide (NEM). Dimerization on non-reducing SDS-PAGE of Tsa1 is normally observed if Tsa1 is active as a peroxidase. Indeed, dimerized Tsa1 species were observed in samples treated with water (likely due to autooxidation, which is common among peroxiredoxins[34]) or H_2O_2 (Fig. 6). In contrast, pronounced Tsa1 dimerization was not observed when the protein was pre-treated with 100 or 1000 μM NEM, suggesting that alkylating agents prevent oxidation and disulfide bond formation by Tsa1. Since modification occurs on active site cysteine residues and this impairs disulfide bond formation in these proteins, it is likely that each of the proteins in the yeast Trx relay are inhibited by monofunctional, as well as bifunctional, electrophiles.

Orthologous Proteins Undergo Cross-Linking in Colorectal Cancer Cells Treated with Bifunctional Electrophiles

Peroxiredoxins and thioredoxins are conserved throughout most biological kingdoms, although thioredoxin reductases fall into two distinct classes between lower and higher eukaryotes.[17,35,36] To determine whether orthologous Trx relay proteins are targets of DVSF

in mammalian cells, we treated RKO cells with DVSF or its monofunctional analog EVSF and looked for alterations in the molecular weights of TrxR1 (the cytosolic Trx reductase), Trx1 (the cytosolic Trx), and Prdx2 (a 2-Cys peroxiredoxin). Each of these proteins underwent dose-dependent cross-linking upon treatment with sub-cytotoxic and toxic doses of DVSF (Fig. 7A).[29] However, cross-linking was not observed in cells treated with EVSF. To test whether other bifunctional electrophiles modify these proteins in a similar manner, we treated RKO cells with toxic doses of DVSF, DAD, DEB, or HN2 (Supp. Fig. 5B, Supp. Table 3)[28] and performed similar immunoblots. While DVSF cross-linked each of these proteins to other cellular proteins most effectively, cross-linking was observed for each of these proteins with some of the bifunctional electrophiles (Fig. 7B). Taken together, these results suggest that orthologous Trx relay proteins in mammalian cells undergo cross-linking in cells treated with bifunctional electrophiles.

Discussion

Collectively, our studies indicate that Trx2, Tsa1, and Trr1, along with their mammalian counterparts, are targets of structurally diverse bifunctional electrophiles and undergo cross-linking to known interaction partners through modification of their redox active Cys residues. These results are in agreement with previous reports that indicate these proteins are intracellular targets of electrophiles derived from endogenous and exogenous sources.[23,25,26,37] Given that the bifunctional electrophiles studied can create complexes of thioredoxins with their redox relay partners,[38] the compounding effect of protein cross-linking within this pathway may elicit a pronounced impact on intracellular redox homeostasis, a property that could be exploited in cancer therapies.[21,22,39] Indeed, several current and prospective cancer therapeutics (including a number of nitrogen mustards, treosulfan, and the natural product piperlongumine) contain two electrophilic centers.[32,33,40-43] These molecules have a number of molecular targets and, in some instances, mediate inter-strand cross-linking within DNA and cross-linking between DNA and proteins.[44-48] In addition, our studies suggest that they may bring about some of their toxic effects by cross-linking proteins to one another, including those proteins in the Trx relay network. In the latter scenario, these electrophiles can directly form adducts with the redox active Cys residues in Trx relay proteins, thereby disrupting this key oxidant defense pathway and potentially promoting redox imbalance.

Each bifunctional electrophile that we tested shows variability in its efficacy at targeting and cross-linking members of the thioredoxin redox relay network. As we noted previously, DVSF more effectively cross-links Trx2 to other proteins than does DAD over a dose range where comparable toxicity is observed.[29] Likewise, when compared with the other bifunctional electrophiles tested, DVSF induces the most pronounced protein-protein cross-linking in peroxiredoxins, thioredoxins, and thioredoxin reductases in both yeast and human cells. The differences in cross-linking between the bifunctional electrophiles tested are likely to depend on a number of factors, including the molecule's membrane permeability, the dose of the electrophile used, its relative electrophilicity, and the sterics through which it interacts with its two distinct targets. For instance, while DVSF cross-links Trx2 to other proteins more effectively than most of the electrophiles tested, DAD can promote a similar amount of cross-linking in the peroxiredoxins as DVSF. Based on our in vitro cross-linking experiments and analysis of available peroxiredoxin crystal structures,[49] we propose that much of the cross-linking observed with Tsa1 in DVSF- and DAD-treated cells is intersubunit cross-linking. Tsa1 and other typical 2-Cys peroxiredoxins form homodimers that orient themselves in head-to-tail configuration; in this orientation, active site Cys residues form intersubunit disulfides upon oxidation of one of these Cys residues.[17,49,50] The spatial arrangement of the two Cys residues is potentially ideal for reacting with structurally compact bifunctional electrophiles like DAD and DVSF, hence explaining why

they can form intersubunit cross-links in Tsa1. Indeed, mutation of either active site Cys residue within Tsa1 to Ala significantly decreases the overall level of cross-linking observed with DVSF or DAD.

While such thiol-reactive cross-linkers may be useful in probing spatial proximity of Cys residues in individual target proteins, our studies also indicate their utility in stabilizing and trapping specific redox-responsive protein-protein interactions in a cell. In a recent study, the fission yeast ortholog of Tsa1, Tpx1, was identified as the major substrate for Trx, provided Tpx1 is not oxidized to the sulfinic or sulfonic acid.[51] In our experiments, we were able to trap the Trx2-Tsa1 interactions using DVSF, suggesting that Tsa1 is likely to be a major Trx2 interacting partner in *S. cerevisiae* as well. Since the peroxiredoxins are highly conserved in terms of sequence and function,[17] we predicted that we would see cross-linked species between peroxiredoxins related to Tsa1 and thioredoxin in lower and higher eukaryotes. However, our results show less cross-linking of Prdx2 to other proteins than we observed with Tsa1. The sole cross-linked species of Prdx2 that forms is likely to be a cross-linked dimer, with little addition of thioredoxin. Sequence variability within the less conserved thioredoxin and thioredoxin reductase proteins may account for this difference between yeast and higher eukaryotes.[36,52]

Despite this, the observation that yeast Trx2 becomes cross-linked to a documented substrate protein in cells treated with bifunctional electrophiles implies that Trx can accommodate these bulky structural perturbations in its active site in order to allow for cross-linking. From a historical standpoint, it is well-established that Trx recognizes protein disulfides in oxidized substrates, reducing these proteins through disulfide exchange reactions.[15] Proteins that are glutathionylated, *S*-nitrosated, and *S*-sulfated are also spatially accommodated and, in some instances, repaired by Trx,[52-56] suggesting that Trx interactions with their binding partners are not disrupted by these diverse thiol modifications. While it is unlikely Trx proteins remove most electrophilic modifications, our studies indicate that Trx can accommodate and interact with its binding partners that are alkylated on redox active Cys residues as well. Since cross-linking by bifunctional electrophiles depends on Cys residue modification in Trx2, our approach represents a novel way to identify putative Trx interaction partners in different organisms and may provide new functional insights into this protein oxidation repair pathway.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

Trx	thioredoxin
Tsa1	yeast thiol-specific antioxidant 1
Trr or TrxR	thioredoxin reductase
DVSF	divinyl sulfone
DAD	diethyl acetylenedicarboxylate
DEB	1,2,3,4-diepoxybutane
HN2	mechlorethamine hydrochloride
EVSF	ethyl vinyl sulfone
NEM	*N*-ethylmaleimide
Prdx2	human peroxiredoxin 2
TAP	tandem affinity purification
IP	immunoprecipitation
IB	immunoblot

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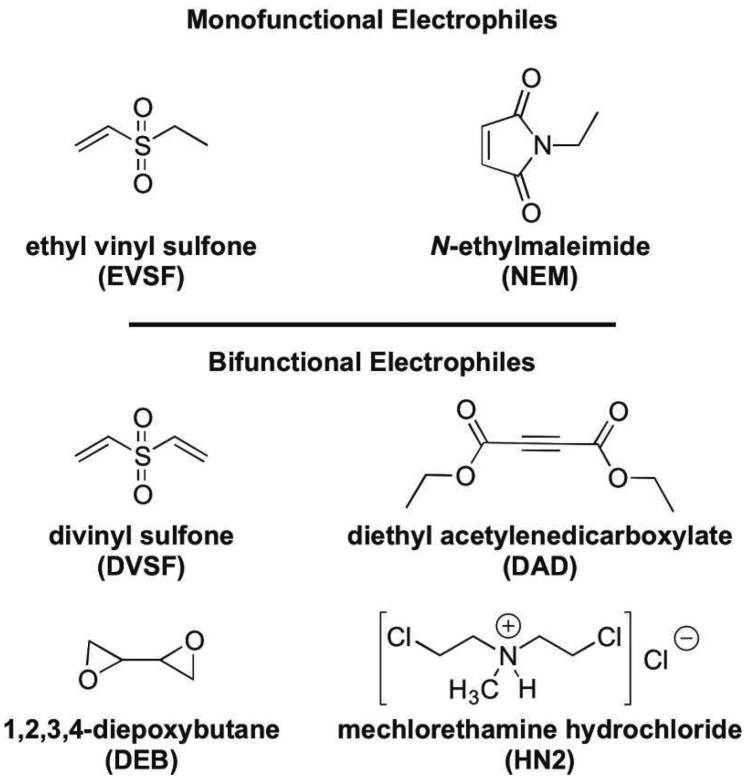

Figure 1.
Structures of Electrophiles Used in This Study.

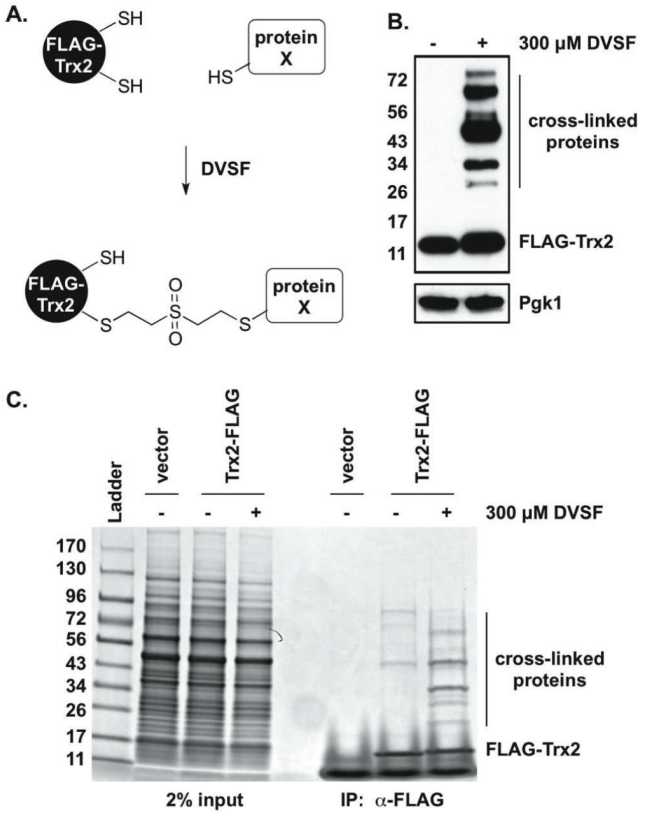

Figure 2. Isolation of Proteins Cross-Linked to Trx2

(A) Scheme depicting the reaction of DVSF with Trx2 and associated proteins. (B) Cross-linking of proteins to FLAG-Trx2 upon DVSF treatment. Yeast cultures expressing FLAG-Trx2 were exposed to DVSF for 1 h. Protein lysates (10 μg/well) were resolved using SDS-PAGE, transferred to PVDF membranes, and probed with antibodies recognizing the FLAG epitope or Pgk1 (loading control). Results are representative of three independent experiments. (C) Immunoprecipitation of FLAG-Trx2 for cross-linked protein identification. Cells containing the empty vector or expressing FLAG-Trx2 were exposed to 300 μM DVSF for 1 h. FLAG-Trx2 was immunoprecipitated from 2 mg of total cell lysate. Following several wash steps and elution with a FLAG peptide, recovered proteins were resolved by SDS-PAGE and visualized with Coomassie Brilliant Blue. Gel pieces from ~10-100 kDa were excised, treated with trypsin, and subjected to LC/MS/MS analysis to identify proteins. Results are representative of two independent experiments.

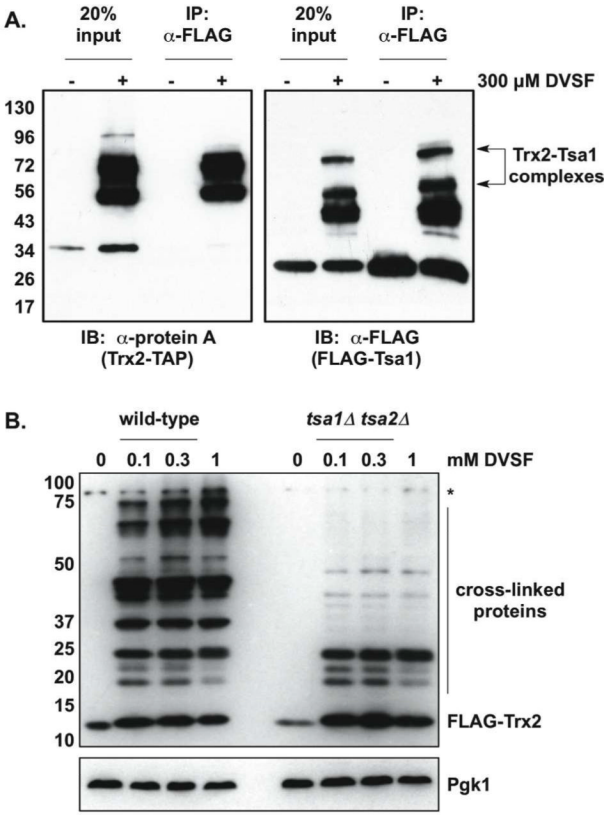

Figure 3. Tsa1 Forms Multiple Cross-Linked Complexes with Trx2

(A) Co-immunoprecipitation of FLAG-Tsa1 with Trx2-TAP following DVSF treatment. Yeast cells expressing FLAG-Tsa1 in a Trx2-TAP strain were treated for 1 h with 300 μM DVSF. FLAG-Tsa1 was immunoprecipitated from 100 μg cellular protein lysates. Immunoprecipitates were resolved using SDS-PAGE, transferred to PVDF membranes, and probed for the presence of the FLAG epitope (to detect FLAG-Tsa1) or protein A (to detect Trx2-TAP) via immunoblot. Results are representative of three independent experiments. (B) Decreased cross-linking of FLAG-Trx2 in yeast lacking Tsa1 and Tsa2. wild-type (BY4741) or *tsa1Δ tsa2Δ* yeast expressing FLAG-Trx2 were exposed to 300 μM DVSF for 1 h. Protein lysates from cultures were subjected to immunoblot for the FLAG tag (to detect changes in cross-linked complexes containing Trx2) or Pgk1 (loading control). An asterisk indicates a non-specific band. Results are representative of three independent experiments.

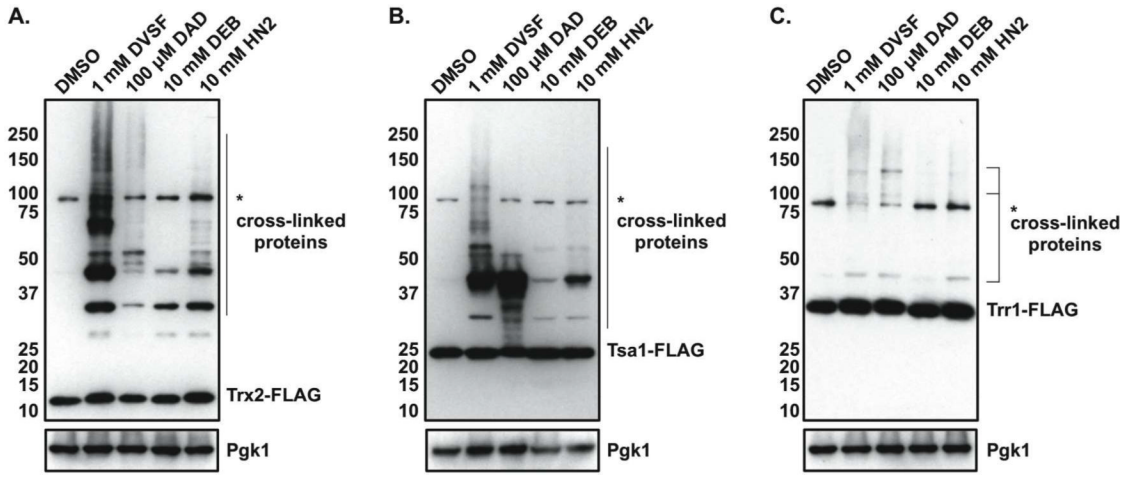

Figure 4. Trx2, Tsa1, and Trr1 Undergo Cross-Linking to Other Proteins in the Presence of Diverse Bifunctional Electrophiles

Yeast cells expressing FLAG-Trx2 (A), FLAG-Tsa1 (B), or FLAG-Trr1 (C) in a wild-type background were treated for 1 h with the indicated bifunctional electrophiles or Me_2SO (vehicle). Protein lysates from treated cells (20 μg) were subjected to immunoblot to detect protein complexes containing the FLAG tag or Pgk1 (loading control). An asterisk indicates a non-specific band. Results are representative of three independent experiments.

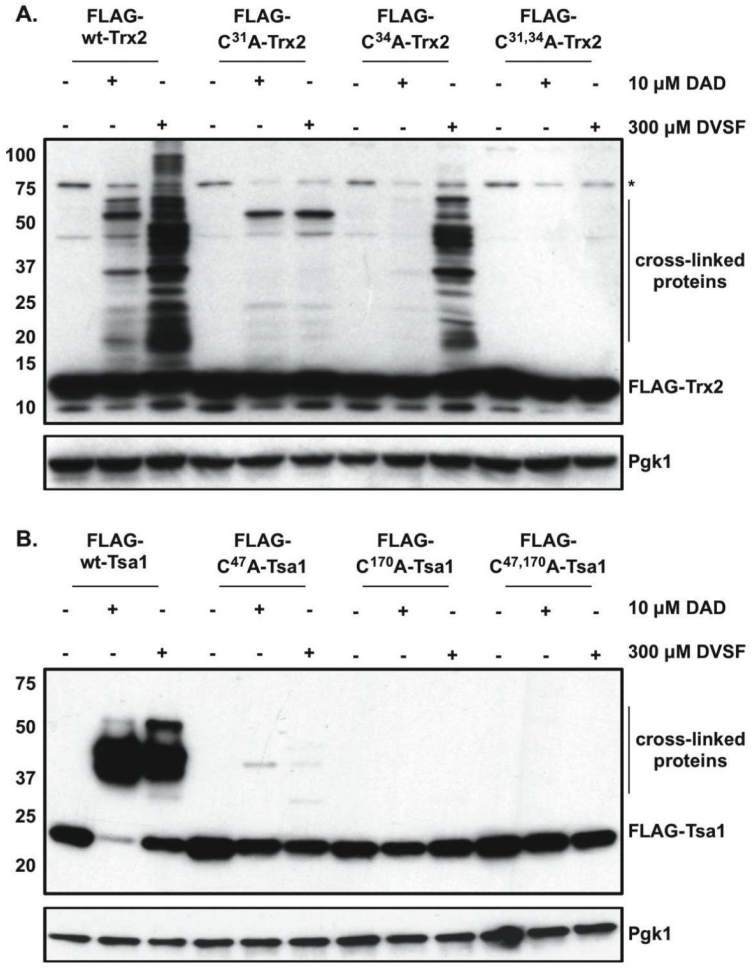

Figure 5. Cross-Linking of Trx2 and Tsa1 by DVSF and DAD Is Dependent on Active Site Cys Residues

Yeast cells expressing wild-type (wt) or mutant FLAG-Trx2 (A) or FLAG-Tsa1 (B) in a wild-type background were treated for 1 h with 10 μM DAD or 300 μM DVSF. Protein lysates from treated cells (20 μg) were subjected to immunoblot to detect protein complexes containing the FLAG tag or Pgk1 (loading control). An asterisk indicates a non-specific band. Results are representative of three independent experiments.

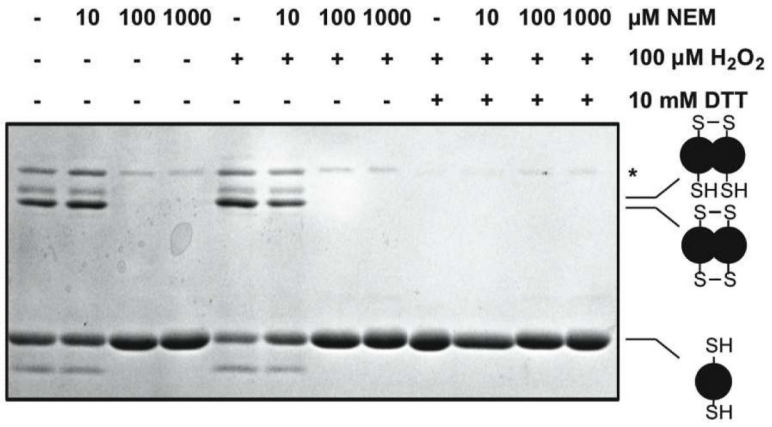

Figure 6. Pre-Treatment of Tsa1 with NEM Blocks Disulfide Bond Formation between Subunits

Recombinant Tsa1 (10 μM) was incubated with increasing concentrations of NEM for 3 h at 37°C. Following removal of NEM, Tsa1 was incubated with 100 mM H_2O_2 or H_2O (control) for 15 min. Reactions were stopped with addition of non-reducing SDS-PAGE buffer with or without DTT. Samples were resolved via SDS-PAGE and visualized with Coomassie blue. Predicted protein complexes formed by oxidation of Tsa1 dimers are shown to the right of the gel. An asterisk indicates an impurity present in all samples. Results are representative of three independent experiments.

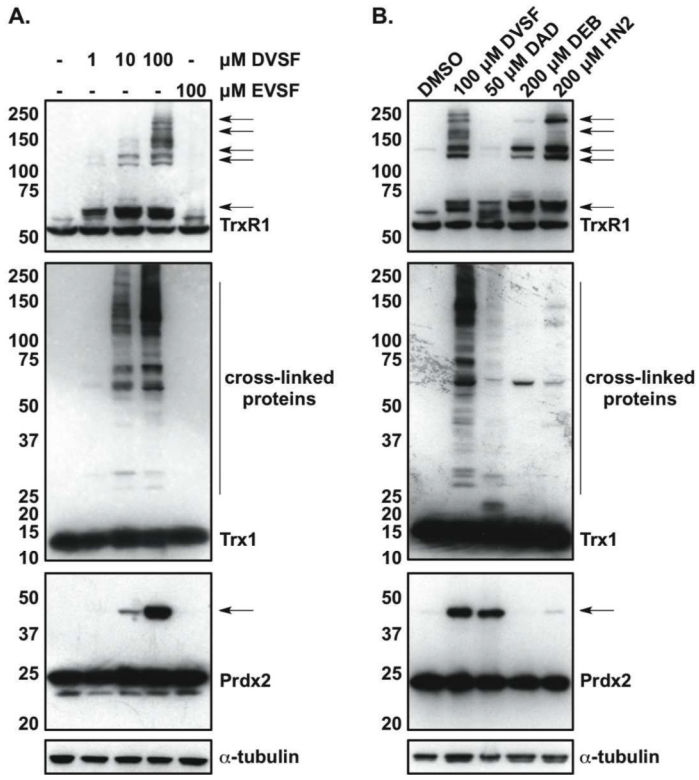

Figure 7. Mammalian TrxR1, Trx1, and Prdx2 Undergo Cross-Linking to Other Proteins in Mammalian Cells Treated with Bifunctional Electrophiles

RKO cells were treated with increasing concentrations of DVSF or EVSF (A) or a panel of bifunctional electrophiles (B) for 1 h. Protein lysates (40 μg) were resolved using SDS-PAGE, transferred to PVDF membrane, and probed with antibodies against TrxR1, Trx2, Prdx2, or α-tubulin (loading control) using immunoblot. Arrows indicate specific cross-linked species formed. Results are representative of three independent experiments.