Chemistry & Biology
Article

Chemical Dissection of an Essential Redox Switch in Yeast

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DOI 10.1016/j.chembiol.2009.01.003

SUMMARY

Saccharomyces cerevisiae **responds to elevated levels of hydrogen peroxide in its environment via a redox relay system comprising the thiol peroxidase Gpx3 and transcription factor Yap1. In this signaling pathway, a central unresolved question is whether cysteine sulfenic acid modification of Gpx3 is required for Yap1 activation in cells. Here we report that cell-permeable chemical probes, which are selective for sulfenic acid, inhibit peroxide-dependent nuclear accumulation of Yap1, trap the Gpx3 sulfenic acid intermediate, and block formation of the Yap1-Gpx3 intermolecular disulfide directly in cells. In addition, we present electrostatic calculations that show cysteine oxidation is accompanied by significant changes in charge distribution, which might facilitate essential conformational rearrangements in Gpx3 during catalysis and intermolecular disulfide formation with Yap1.**

INTRODUCTION

All organisms have evolved cellular responses to monitor and adapt to adverse and changing environmental conditions. In most cases, the stress response is initiated at the genetic level and, ultimately, involves the synthesis of proteins that serve a protective function (Amundson et al., 1999; Gasch et al., 2000; Hua et al., 2004; Paulding et al., 2002; Prasad et al., 2004). One type of response mechanism is triggered by reactive oxygen species (ROS), including hydrogen peroxide (H_2O_2), superoxide ($O_2^{\bullet -}$), and hydroxyl radical (OH•), which are toxic because of their ability to damage DNA and proteins (Miller and Chang, 2007). As a result, aerobic organisms have defense mechanisms to protect against ROS. The oxidative stress response has been well characterized in *Escherichia coli* and *Salmonella typhimurium* where transcription factors such as OxyR (Christman et al., 1985; Kim et al., 2002; Lee et al., 2004; Storz et al., 1990; Zheng et al., 1998, 2001) and SoxR/SoxS (D'Autreaux and Toledano, 2007; Nunoshiba et al., 1992; Tsaneva and Weiss, 1990; Wu and Weiss, 1991) upregulate the expression of genes involved in ROS metabolism. Genetic screens have also enabled the identification of transcription factors that regulate antioxidant systems in yeast (Krems et al., 1995; Schnell and Entian, 1991).

In *Saccharomyces cerevisiae* the transcription factor Yap1 is a central regulator for the oxidative stress response (D'Autreaux and Toledano, 2007; Stone and Yang, 2006). Yap1 is a basic leucine zipper (bZIP) transcription factor and its DNA-binding domain exhibits homology to members of the mammalian Jun family of proteins (Harshman et al., 1988; Moye-Rowley et al., 1989). Yap1 contains a noncanonical leucine-rich nuclear export signal embedded within its C-terminal, cysteine-rich domain (c-CRD) composed of residues Cys598, Cys620, and Cys629 (Delaunay et al., 2000). A second cysteine-rich domain in Yap1 is located within the N-terminal region and is composed of residues Cys303, Cys310, and Cys315 (Delaunay et al., 2000). Yap1 is essential for yeast survival under conditions of oxidative stress (Krems et al., 1995; Schnell and Entian, 1991) as well as for cellular resistance to diamide, electrophiles, and cadmium (Azevedo et al., 2003; Coleman et al., 1999; Schnell and Entian, 1991; Wemmie et al., 1994; Wu et al., 1993). In response to hydrogen peroxide, Yap1 stimulates the expression of ~100 genes including the *TRX2* gene, which encodes thioredoxin; *GSH1*, which encodes γ-glutamylcysteine synthetase involved in glutathione biosynthesis; and *GLR1,* which encodes glutathione reductase (Gasch et al., 2000; Kuge and Jones, 1994).

Responding to changes in cellular ROS is critical for cell viability and, as a result, there has been significant interest in elucidating the molecular mechanism(s) of transcription factor activation by oxidative stress (Delaunay et al., 2000, 2002; Koh et al., 2007; Lee et al., 2004, 2007; Storz et al., 1990; Zheng et al., 1998). In 1997, Kuge and colleagues reported that oxidative stress caused Yap1 to translocate from the cytosol to the nucleus, and that localization was mediated by conserved cysteine residues in the c-CRD (Kuge et al., 1997). In vitro studies reported by Delaunay et al. demonstrated that peroxide stress triggered disulfide formation between Cys303 and Cys598 (Delaunay et al., 2000). The structural basis of Yap1 activation was revealed in a solution structure, which showed that in its active, oxidized form the nuclear export signal in the c-CRD of Yap1 was masked by disulfide-mediated interactions (Wood et al., 2004). Additional studies demonstrated that a second protein was required for peroxide-induced Yap1 activation, which was identified as the non-heme peroxide-scavenging enzyme Gpx3 (Delaunay et al., 2002). Notably, a yeast mutant with substitution of Gpx3 Cys36 to serine could not activate Yap1 and expression of Yap1 Cys303Ala from a centromeric

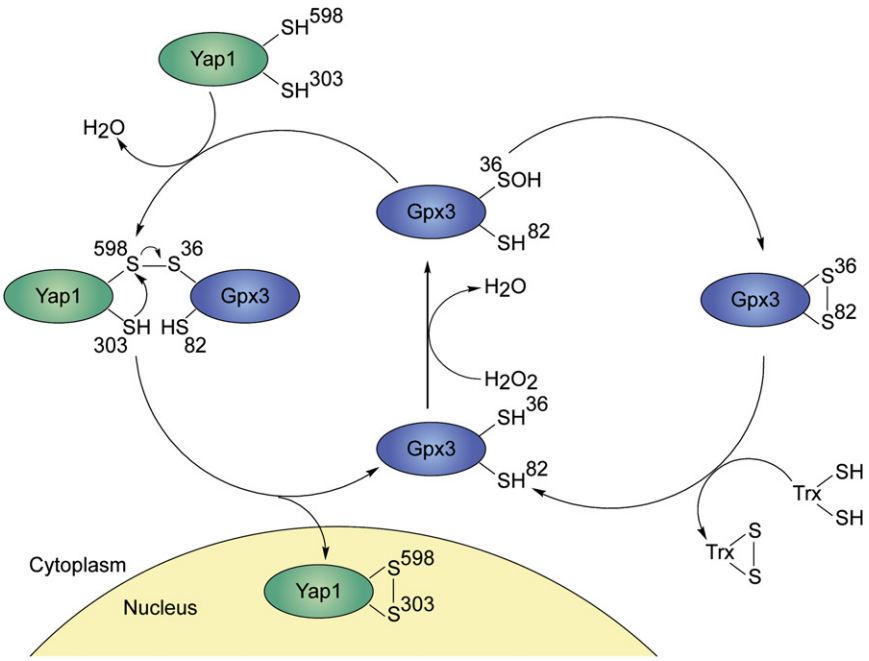

Figure 1. Proposed Molecular Mechanism for the Yap1-Gpx3 Redox Relay

Gpx3 is hypothesized to transmit a stress signal to Yap1 through oxidation of its catalytic cysteine (Cys36) to sulfenic acid. Next, the Gpx3 Cys36 sulfenic acid condenses with Cys598 in Yap1 to form the Yap1-Gpx3 intermolecular disulfide. Thiol-disulfide exchange with Yap1 Cys303 generates the Cys303-Cys598 intramolecular disulfide in Yap1. Formation of this disulfide masks the nuclear export signal and results in nuclear accumulation of the transcription factor. Figure adapted from (D'Autreaux and Toledano, 2007).

low-copy number plasmid stabilized a disulfide-linked complex with Gpx3 (Delaunay et al., 2002).

From these collective studies, Delaunay et al. proposed the mechanism for Gpx3-mediated Yap1 activation that is summarized in Figure 1 (Delaunay et al., 2002; D'Autreaux and Toledano, 2007). In this model, the Gpx3 active site cysteine (Cys36) is oxidized to a sulfenic acid, which can react with the resolving cysteine in Gpx3 (Cys82) or with Yap1 Cys598 to form an intra- or intermolecular disulfide, respectively. In the latter pathway, thiol-disulfide exchange leads to a disulfide between Cys303 and Cys598 in Yap1 and nuclear accumulation of the transcription factor. Although a cysteine sulfenic acid modification is central to these competing pathways, it has yet not been determined whether this posttranslational modification is essential for Yap1 activation in cells or how Gpx3 Cys36 and Cys82, which are predicted to be separated by more than 13 Å in the reduced state, come into proximity to form a disulfide (Poole et al., 2004; Stone and Yang, 2006).

In this report, we take a chemical biology approach to address the role of cysteine sulfenic acid modification in Yap1 activation. Here, we present four key results. First, we demonstrate that cell-permeable chemical probes, which covalently modify sulfenic acids, intercept the Yap1-Gpx3 redox relay and inhibit peroxide-induced nuclear localization of Yap1. Second, we show that disruption of the Yap1-Gpx3 relay is associated with peroxide-dependent labeling of the sulfenic acid intermediate in wild-type Gpx3, in vitro and directly in cells. Third, we demonstrate that a probe for sulfenic acid inhibits formation of the Yap1-Gpx3 intermolecular disulfide in vivo. Taken together, these studies demonstrate the essential role of sulfenic acid posttranslational modification in the Yap1-Gpx3 redox relay. Finally, electrostatic calculations indicate that cysteine oxidation is accompanied by a significant amount of negative charge localization to the sulfenate oxygen, which could be exploited by Gpx3 to carry out the conformational change required for intramolecular disulfide formation during catalysis as well as intermolecular disulfide formation with Yap1.

RESULTS AND DISCUSSION

Sulfenic Acid-Specific Chemical Probes Inhibit Yap1 Nuclear Localization

The mechanism outlined in Figure 1 predicts that the sulfenic acid intermediate, which forms at Gpx3 Cys36, during the catalytic cycle is essential for Yap1 activation and nuclear localization. To test this model, we reasoned that a small molecule that is cell permeable and chemically selective for sulfenic acid could trap the Gpx3 sulfenic acid modification. Therefore, if the Gpx3 sulfenic acid modification is essential for Yap1 nuclear localization, yeast treated with a sulfenic acid-specific probe should not exhibit peroxide-induced nuclear localization of the transcription factor. Alternatively, if the Gpx3 sulfenic acid intermediate is not essential, the probe should not inhibit nuclear localization of Yap1.

To distinguish between these models, we used a green fluorescent protein (GFP)-tagged Yap1 yeast strain (Huh et al., 2003) and fluorescence microscopy to monitor the effect of sulfenic acid reactive probes on Yap1 localization. In untreated samples of logarithmically growing yeast cells, Yap1 showed primarily cytosolic localization (Figures 2A and 2B). After hydrogen peroxide stimulation, Yap1 accumulated in the nucleus, reaching maximal nuclear translocation within 5 min of peroxide treatment (Figures 2A and 2B). After ~30 min, fluorescently labeled protein moved back to the cytosol (Figures 2A and 2B). These kinetics are consistent with published data describing peroxide-dependent Yap1 nuclear translocation (Delaunay et al., 2000; Kuge et al., 1997).

To trap the sulfenic acid modification in Gpx3 in cells and prevent thiol-disulfide exchange with Yap1, we used dimedone (5,5-dimethyl-1,3-cyclohexadione), a cell-permeable and nucleophilic small molecule that is chemically selective for sulfenic acids (Allison, 1976; Benitez and Allison, 1974; Poole et al., 2005, 2007; Reddie et al., 2008; Seo and Carroll, 2009). In this reaction, dimedone reacts with the electrophilic sulfur atom in sulfenic acid to form a stable, thioether bond (Figure 3A). Dimedone alone had no effect on Yap1 distribution in nonstimulated cells (data not shown), but completely blocked its nuclear translocation in peroxide-treated cells (Figures 3B and 3C). Peroxide-dependent nuclear localization of Yap1 could be completely restored by diluting dimedone-treated yeast in fresh medium

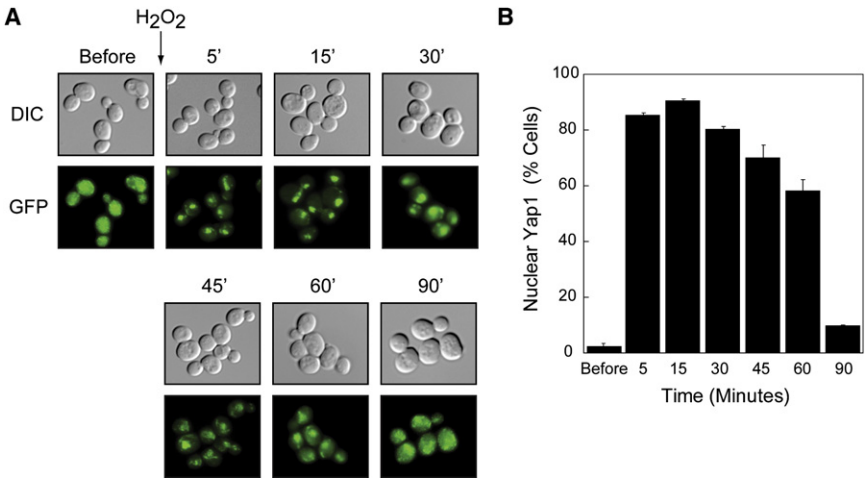

Figure 2. Analysis of Yap1-GFP Cellular Localization upon Activation by Hydrogen Peroxide

(A) Exponentially growing yeast with a chromosomal copy of Yap1 fused to GFP (Yap1-GFP) were treated with hydrogen peroxide (400 μM) during the indicated period and analyzed for GFP staining.

(B) Kinetics of Yap1-GFP nuclear localization. Cells (n = 200–300) from (A) were scored for nuclear accumulation of Yap1-GFP. Error bars represent standard deviations from two separate experiments.

lacking inhibitor and culturing cells for 16 hr (see Figure S1 available online). Because the protein-dimedone adduct is irreversible, these data suggest that the redox relay is restored by degrading modified Gpx3 and biosynthesis of new thiol peroxidase. The rate of turnover for Gpx3 protein has not been reported, but the half-life for Gpx2 in budding yeast is 168 min (Belle et al., 2006). Based on this estimate, complete degradation (e.g., five-half lives) of modified Gpx3 would take approximately 14 hr, consistent with our observations. Taken together, these data demonstrate that a sulfenic acid modification is required for peroxide-induced Yap1 nuclear accumulation, directly in living cells.

Trapping the Gpx3-Sulfenic Acid Modification In Vivo

The above results are consistent with the model proposed in Figure 1. However, the data presented in Figure 3 do not indicate whether Gpx3 is modified by sulfenic acid-specific probes in peroxide-treated cells. To show that inhibition of Yap1 nuclear localization is accompanied by peroxide-dependent tagging of Gpx3 in cells we used DAz-1, a sulfenic acid-specific probe that we have recently developed in our lab (Figure 3A) (Reddie et al., 2008; Seo and Carroll, 2009). Based on the dimedone scaffold, this probe is also functionalized with an azide chemical handle that can be selectively detected with phosphine or alkyne-based reagents via the Staudinger ligation or click chemistry for detection of modified proteins (Agard et al., 2006) (Figure 4A). Peroxide-dependent Yap1 nuclear accumulation was also suppressed in DAz-1-treated cells, as expected (data not shown).

To facilitate enrichment of Gpx3, a relatively low-abundance protein, we inserted the FLAG epitope into chromosomal Gpx3 (Figure S2A). Control experiments verified that the tag did not disrupt the Yap1-Gpx3 redox relay (Figures S2B and S2C) or the ability of sulfenic acid-specific chemical probes to inhibit peroxide-induced Yap1 nuclear accumulation (Figures S2D and S2E). To probe for sulfenic acid modification of Gpx3 in vitro,

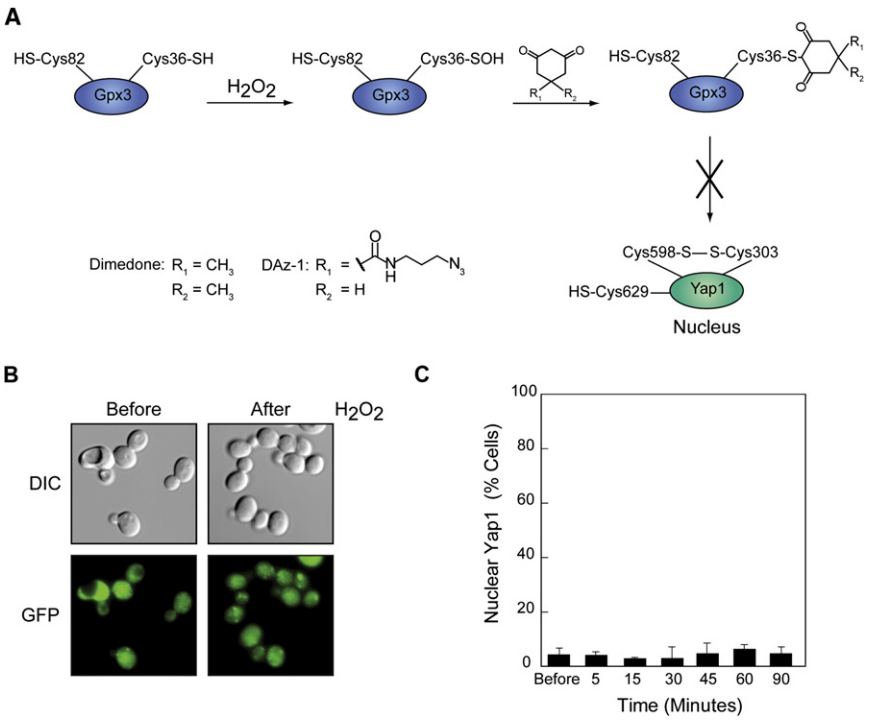

Figure 3. Dimedone Inhibits Peroxide-Dependent Nuclear Localization of Yap1-GFP

(A) Dimedone and DAz-1 selectively modify sulfenic acids (Allison, 1976; Poole et al., 2005, 2007; Reddie et al., 2008; Seo and Carroll, 2009). Reaction of either probe with Gpx3 Cys36 sulfenic acid will form a stable adduct and, based on the model in Figure 1, is predicted to inhibit peroxide-dependent nuclear localization of Yap1.

(B) Yap1-GFP remains in the cytoplasm when cells are exposed to dimedone. Exponentially growing yeast were treated with dimedone (50 mM) and hydrogen peroxide (400 μM) for 15 min at 30°C and analyzed for GFP staining.

(C) Kinetics of Yap1-GFP nuclear localization in yeast exposed to dimedone. Cells (n = 200–300) from (B) were scored as in Figure 2B. Error bars represent standard deviations from two separate experiments.

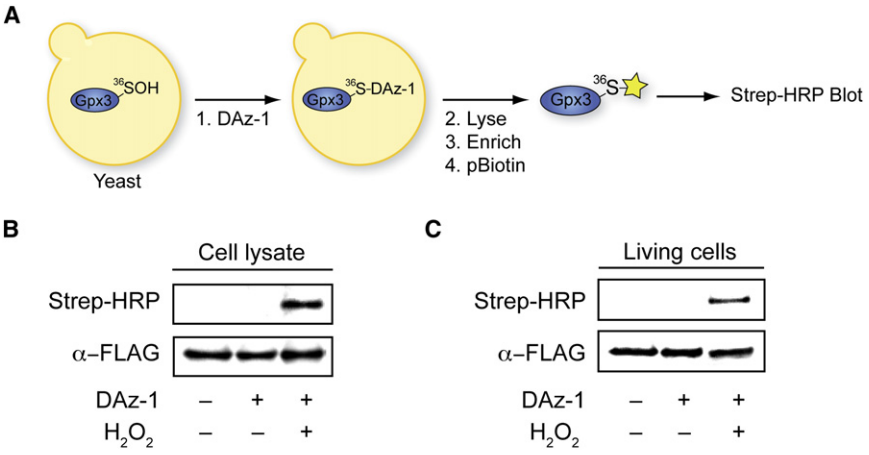

Figure 4. The Sulfenic Acid Azido Probe DAz-1 Traps the Gpx3 Sulfenic Acid Intermediate in Lysate and Yeast Cells

(A) Strategy to trap the Gpx3 sulfenic acid intermediate with DAz-1.

(B) The Gpx3-FLAG sulfenic acid intermediate is trapped by DAz-1 in cell lysate. Extracts (100 μg protein) produced from yeast cells, untreated or exposed to hydrogen peroxide (10 μM), were incubated in the presence or absence of DAz-1 (1 mM) for 1 h at 37°C. After DAz-1 labeling, Gpx3-FLAG was immunoprecipitated with FLAG M2 affinity resin, reacted with p-biotin (250 μM), resolved under reducing conditions, and analyzed by HRP-streptavidin western blot.

(C) The Gpx3-FLAG sulfenic acid intermediate is trapped by DAz-1 directly in cells. Exponentially growing yeast not treated or treated with DAz-1 (50 mM) were exposed to hydrogen peroxide (400 μM) for 10 min at 30°C. Extracts were prepared, Gpx3-FLAG was immunoprecipitated with FLAG M2 affinity resin, and samples were analyzed as in (B).

whole cell yeast lysate was not treated or treated with DAz-1, in the presence or absence of hydrogen peroxide. Subsequently, Gpx3 was immunoprecipitated and conjugated to phosphine-biotin (p-Biotin). Samples were analyzed under reducing conditions and DAz-1 labeling was detected by streptavidin-horseradish peroxidase (HRP) western blot (Figure 4A). Gpx3 showed peroxide-dependent labeling by DAz-1 (Figure 4B). Control reactions done in the absence of DAz-1 or hydrogen peroxide showed no labeling, as expected (Figure 4B). Next we investigated whether DAz-1 could trap the Gpx3 sulfenic acid intermediate directly in cells. For these experiments, cells were not treated or treated with DAz-1, in the presence or absence of hydrogen peroxide. Gpx3 showed peroxide-dependent labeling by DAz-1 (Figure 4C). As before, no signal was observed in the absence of DAz-1 or hydrogen peroxide (Figure 4C). Collectively, these data show that DAz-1 can trap the Gpx3-sulfenic acid intermediate in lysate and directly in cells.

A sulfenic acid intermediate has been detected at the active site in thiol peroxidases when the resolving cysteine is not present to generate the disulfide bond (Ma et al., 2007; Poole et al., 2007). To our knowledge, however, chemical trapping of the sulfenic acid intermediate in a wild-type thiol peroxidase has not yet been reported. To provide additional evidence that dimedone can trap the reactive sulfenic acid intermediate in Gpx3, we investigated peroxide-dependent disulfide formation in recombinant his-tagged Gpx3, in the presence or absence of dimedone. For these experiments, we analyzed Gpx3 under nonreducing conditions, which permit the oxidized and reduced forms of Gpx3 to be distinguished by their electrophoretic mobility (Delaunay et al., 2002). Treatment with hydrogen peroxide converted Gpx3 from its reduced to its oxidized form (Figure 5A, lanes 1 and 2). However, in the presence of dimedone, the majority of Gpx3 remained in the reduced state (Figure 5A, lane 3). This observation is consistent with covalent modification of the Gpx3 Cys36 sulfenic acid intermediate by dimedone and inhibition of intramolecular disulfide formation between Cys36 and Cys82.

Next, we investigated whether DAz-1 could trap the sulfenic acid intermediate in recombinant wild-type and mutant Gpx3 protein. Peroxide-dependent DAz-1 labeling of wild-type Gpx3 was observed (Figure 5B, lanes 1 and 2), analogous to results obtained with Gpx3-FLAG in yeast lysate and cells. Additional studies performed with Gpx3 Cys36Ser and Gpx3 Cys36Ser Cys64Ser (Figure S3) also indicate that DAz-1 labels the active site Cys36, consistent with previous results obtained using NBD-Cl (Ma et al., 2007). Finally, the intensity of DAz-1 labeling

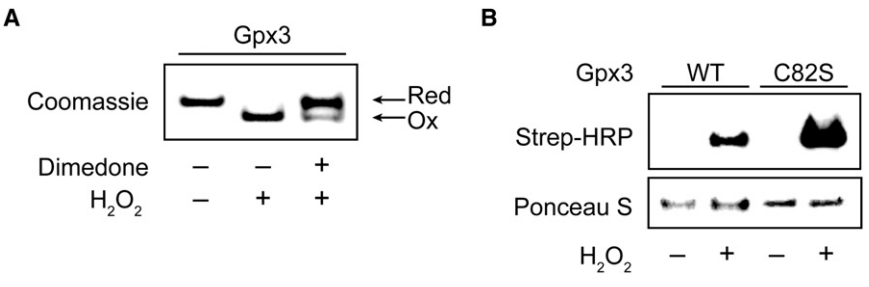

Figure 5. Dimedone Treatment Inhibits Formation of the Intramolecular Disulfide in Recombinant Wild-Type and or Cys82Ser Gpx3

(A) Analysis of the in vitro redox state of recombinant Gpx3. Recombinant wild-type Gpx3 (60 μM), untreated or exposed to hydrogen peroxide (60 μM), was incubated in the presence or absence of dimedone (50 mM). Samples were resolved under nonreducing conditions and visualized by Coomassie blue stain. The oxidized and reduced forms of Gpx3 are indicated by arrows.

(B) DAz-1 traps the sulfenic acid intermediate in wild-type and mutant Gpx3. Wild-type or Cys82Ser Gpx3 (50 μM), untreated or exposed to hydrogen peroxide (100 μM), were incubated in the presence or absence of DAz-1 (10 mM) for 15 min at 37°C. After DAz-1 labeling, samples were treated with p-biotin (250 μM), resolved under reducing conditions, and analyzed by HRP-streptavidin western blot.

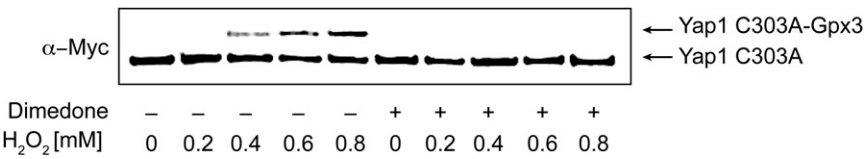

Figure 6. Dimedone Inhibits Formation of the Yap1-Gpx3 Intermolecular Disulfide In Vivo

Exponentially growing yeast, carrying Myc-Yap1 Cys303Ala, untreated or exposed to dimedone (50 mM), were treated with hydrogen peroxide at the concentrations indicated for 2 min. NEM-blocked extracts were prepared as described in Experimental Procedures, resolved by nonreducing SDS-PAGE and analyzed by western blot using antibodies against the Myc epitope. Myc-Yap1 Cys303Ala and the Myc-Yap1 Cys303Ala-Gpx3 mixed disulfide are indicated by arrows.

was increased in the Gpx3 Cys82Ser mutant, as expected (Figure 5B, lanes 3 and 4).

In a previous study, Ellis and Poole investigated sulfenic acid formation in the bacterial thiol peroxidase AhpC, but failed to observe reactivity with dimedone during the catalytic cycle (Ellis and Poole, 1997). In these experiments, it is possible that the sulfenic acid in wild-type AhpC was not trapped by dimedone due to the low concentration of probe employed in these experiments (Ellis and Poole, 1997). Moreover, a more recent study reported by Poole and coworkers shows that the sulfenic acid intermediate in AhpC Cys165Ser reacts more slowly with a dimedone analog, relative to an oxidized cysteine protease (Poole et al., 2007). Hence, rates of covalent modification might depend on the surrounding microenvironment of the sulfenic acid and vary from protein to protein.

Dimedone Blocks Formation of the Yap1-Gpx3 Intermolecular Disulfide In Vivo

Previously, Toledano and coworkers demonstrated that expression of Yap1 Cys303Ala stabilized a disulfide-linked complex with Gpx3 (Delaunay et al., 2002). Given this finding and the data presented in Figures 2–5, we reasoned that covalent modification of the Gpx3 sulfenic acid intermediate by dimedone should inhibit formation of the Yap1-Gpx3 intermolecular disulfide in vivo. To test this hypothesis, we generated a plasmid encoding Yap1 Cys303Ala with an N-terminal Myc epitope and transformed this construct into yeast. In subsequent steps, we challenged yeast with hydrogen peroxide, in the presence or absence of dimedone. After processing, cellular proteins were resolved under nonreducing conditions and Yap1-Gpx3 complex formation was monitored by western blot (Figure 6 and Figure S4).

In the absence of hydrogen peroxide or at a low concentration of the oxidant (200 μM), the western blot showed only a single band corresponding to Yap1 Cys303Ala (Figure 6, lanes 1 and 2). However, as the concentration of hydrogen peroxide was raised (400–800 μM), we observed a second band in the western blot, which increased in intensity and migrated approximately 25 kDa higher than Yap1 Cys303Ala (Figure 6, lanes 3–5). As predicted for the Yap1-Gpx3 complex, the higher molecular weight species disappeared when samples were analyzed under reducing conditions (data not shown) and was not observed in a *gpx3*-null strain (Figure S4). To test whether dimedone could inhibit formation of the Yap1-Gpx3 complex, we conducted side-by-side experiments in the presence of the chemical probe. Notably, the higher molecular weight Yap1-Gpx3 complex was not observed in dimedone-treated cells (Figure 6, lanes 6–10). Likewise, the Yap1 Cys303Ala-Gpx3 complex could be selectively immunoprecipitated from peroxide-treated cells, but was not formed in dimedone-treated samples (Figure S5). Together, these data show that dimedone inhibits the formation of the Yap1-Gpx3 intermolecular disulfide in vivo.

Sulfenic Acid Formation: A General Mechanism for Conformational Change

A recent crystal structure of the peroxidase Gpx5 from *Populus trichocarpa x deltoids* (PtGPX5) determined that the catalytic and resolving cysteines are located 21 Å apart in the reduced enzyme (Koh et al., 2007). Likewise, structure homology modeling predicts that Cys36 and Cys82 in Gpx3 are separated by 13 Å (Poole et al., 2004). Therefore, the transition between the reduced and oxidized states is accompanied by significant conformational changes in this family of peroxidases. In their analysis of the PtGpx5 structures, Koh and colleagues propose that during the catalytic cycle, deprotonation of Cys92 to form the thiolate anion destabilizes adjacent structural elements, and thereby facilitates conformational change (Koh et al., 2007). However, because the catalytic cysteine in the peroxidase is characterized by a low pK_a (Poole et al., 2004), this residue is constitutively deprotonated at physiological pH. Therefore, it seems unlikely that the thiolate mediates conformational change during the catalytic cycle. Rather, we hypothesize that oxidation of the thiolate to the sulfenic acid, which is also expected to be deprotonated at physiological pH (Poole and Ellis, 2002), is responsible for accelerating the rate of intramolecular disulfide formation.

To investigate changes in charge-density distribution that occur when a thiolate is oxidized to a sulfenate, we generated potential energy surfaces for these functional groups (Figure 7A). These calculations show that the charge-density distribution differs dramatically between these states. Notably, cysteine oxidation is accompanied by a significant localization of negative charge to the sulfenate oxygen atom. Therefore, when surrounded by hydrophobic and electronegative residues (Figure 7B) formation of the sulfenate anion in the Gpx3 active site might promote conformational rearrangement via electrostatic repulsion, which is required for intramolecular disulfide formation during catalysis and for intermolecular disulfide formation with Yap1.

SIGNIFICANCE

Reduction and oxidation comprise an important class of posttranslational modifications. In this context, the thiol side chain of cysteine is most sensitive to redox transformations and can occur in a variety of oxidation states. Among these, the thiol and the disulfide are best known, but oxygen derivatives such as sulfenic (RSOH), sulfinic (RSO_2H), and sulfonic (RSO_3H) acid are observed in a growing number of proteins, and are proposed to regulate a wide variety of phenomena such as catalysis, metal binding, protein turnover, and signal transduction (Poole and Nelson, 2008;

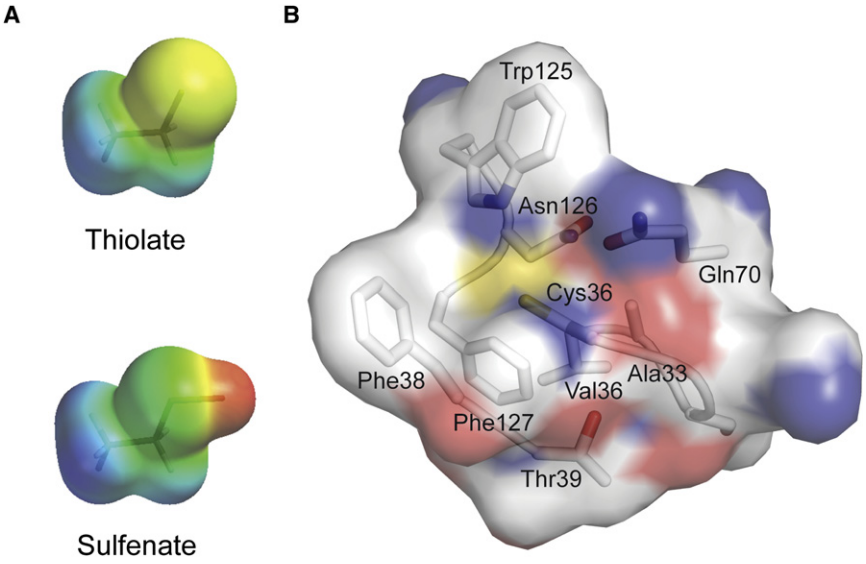

Figure 7. Cysteine Oxidation to Sulfenic Acid as a Mechanism for Conformational Change

(A) Electrostatic potential surface of cysteine in the thiolate and sulfenate forms. The surfaces depict the highly delocalized negative charge of a nucleophilic thiolate and the accumulation of negative charge on the sulfenate oxygen atom. Electrostatic potential surfaces were generated using Spartan 06 (Wavefunction, Inc.). Color gradient: red corresponds to most negative and blue corresponds to most positive.

(B) Structure-homology-based model of the yeast Gpx3 active site depicted with negative (red) and positive (blue) electrostatic surface potentials. Hydrophobic residues Ala33, Val34, Phe38, Trp125, and Phe127 surround the sulfur atom of Cys36 (yellow). The thiol functional group is also in proximity to amide functional groups from Gln70 and Asn126. This model was generated using the Swiss Model program (Guex and Peitsch, 1997) with human Gpx3 (Protein Data Bank code 2r37), related to yeast Gpx3 by ~36% identify, as the structural template. The electrostatic surface potential and figure were generated in Pymol (http://pymol.sourceforge.net).

Reddie and Carroll, 2008). This study constitutes the first direct evidence that cysteine oxidation to sulfenic acid in the thiol peroxidase Gpx3 is essential for yeast to sense oxidative stress and, more broadly, sheds light on the growing roles of sulfenic acid modifications in biology. From a chemical perspective, this work highlights the utility of cell-permeable, small-molecule probes to investigate redox-regulated signal transduction in living cells (Miller and Chang, 2007; Poole and Nelson, 2008; Reddie and Carroll, 2008). Finally, this work also contributes to our molecular understanding of how oxidative cysteine modification and accompanying changes in electrostatic charge distribution might be exploited to facilitate conformational change in proteins.

EXPERIMENTAL PROCEDURES

Strains and Growth Conditions

The *S. cerevisiae* strain ATCC-201388 (*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*) containing GFP-modified Yap1 was used in all experiments, with the exception of the *Δgpx3*, which is in BY4742 (*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 can1-100*). Cells were grown at 30°C in YPD (1% yeast extracts, 2% bactopeptone, and 2% glucose) or SC-Ura media containing 2% glucose. The Gpx3-FLAG strain was derived from the Yap1-GFP strain. The FLAG epitope was appended to Gpx3 in the yeast chromosome as described previously (Gelbart et al., 2001). In brief, primers 5′-AAACCTTCTTCGTTGTCCGAAACCATCGAAGAACTTTT GAAAGAGGTGG AAAGGGAACAAAAGCTGGAG-3′ and 5′-AAATATAAAA GAAAACTAAGCTTTACCT AACTTCAAAAGAAGAAGACCTGCCTATAGGGC GAATTGGGT-3′ were used to amplify a 3xFLAG/Kan cassette with regions of homology to *gpx3* from the p3FLAG-KanMX plasmid. The polymerase chain reaction (PCR)-amplified product was transformed into the Yap1-GFP yeast strain (Gietz and Schiestl, 1991; Gietz and Woods, 2001). Transformants were selected on YP-Gal plates supplemented with G418 (200 mg ml^{-1}). Chromosomal tagging was verified by PCR and anti-FLAG western blot.

Cloning, Expression, and Purification of Recombinant Gpx3

Yeast genomic DNA was isolated from the Yap1-GFP yeast strain as described elsewhere (Rose et al., 1990). *gpx3* was amplified by PCR from yeast genomic DNA using the following primers: 5′-TTTATCGGATCCATGTCAGAATTCTA TAAGCTAGCACCT-3′ and 5′-ACCTGCCTCGAG CTATTCCACCTCTTTCAA AAGTTCTTC-3′. pRSETa-6xHis-Gpx3 was constructed by subcloning *gpx3* into the BamHI and XhoI sites of pRSETa (Invitrogen). pRSETa-6xHis-Gpx3 Cys36Ser, pRSETa-6xHis-Gpx3 Cys82Ser, and pRSETa-6xHis-Gpx3 Cys36Ser Cys64Ser were generated using site-directed PCR mutagenesis. Wild-type, Cys82Ser, Cys36Ser, and Cys36Ser Cys64Ser Gpx3 were purified from *Escherichia coli* strain BL21(DE3) pLysS as previously described (Delaunay et al., 2002; Ma et al., 2007).

Construction Myc-Yap1 Cys303Ala

yap1 was amplified by PCR from yeast genomic DNA using the following primers: 5′-TAAACCTCTAGAATGAGTGTGTCTACCGCCAAGAGGTC-3′ and 5′-CCCGCTCTCG AGTTAGTTCATATGCTTATTCAAAGCTA–3′. *yap1* was then subcloned into pCR4 (Invitrogen). pCR4-Yap1 Cys303Ala was generated by site-directed PCR mutagenesis. Myc-Yap1 Cys303Ala was generated in two steps by PCR. The first PCR product was generated from the pCR4-Yap1 Cys303Ala template using the following primers: 5′-GATTTCCGAAG AAGACCTCATGAGTGTGTCTACCGCC-3′ and 5′-GTTCATATGCTT ATTCA AAGCTAATTGAACGTCTTCTGC-3′. The product of this reaction was then used to generate the complete Myc-Yap1 Cys303Ala fragment using the following primers: 5′-AAGCTTATGGAACAGAAGTTGATTTCCGAAGAAGAC CTC-3′ and 5′-GTTCATATGC TTATTCAAAGCTAATTGAACGTCTTCTGC-3′. Finally, the Myc-Yap1 Cys303Ala PCR product was digested with HindIII and XhoI and subcloned into the multiple cloning region of p416-TEF (Mumberg et al., 1995).

Stock Solutions of Sulfenic Acid Probes

Dimedone was prepared in dimethyl sulfoxide (DMSO) at a final concentration of 1.1 M. DAz-1 was synthesized as previously described (Seo and Carroll, 2009) and prepared as a 50:50 mixture of DMSO and 0.1 M Bis-Tris HCl (pH 7.0) at a final concentration of 0.25 M. Chemical probes were added directly to culture or reactions in vitro.

Yeast Culture with Sulfenic Acid Probes

Exponentially growing yeast were treated with dimedone or DAz-1 (50–100 mM) and cultured for 30–60 min before treatment with hydrogen peroxide (400 μM). At the indicated times, cells were fixed by incubating for 15 min at room temperature (rt) with 4% (w/v) paraformaldehyde with rocking. To restore peroxide-dependent nuclear localization of Yap1, dimedone-treated cells

were diluted 10,000-fold in fresh media lacking probe, grown to saturation (16 hr), and rechallenged with hydrogen peroxide (400 μM). Samples were fixed and analyzed as described above.

Fluorescence Microscopy

Exponentially growing yeast (1 ml) were harvested and washed twice with 0.1 M KH_2PO_4 (pH 6.6) and stored at 4°C in the same buffer. For oxidized samples, exponentially growing yeast (1 ml) were exposed to hydrogen peroxide (400 μM) and samples were fixed at various time points, as described above. Yap1-GFP nucleocytoplasmic localization was analyzed with a Nikon Eclipse 80i fluorescent microscope equipped with a Photometrics CoolSnap ES^2 cooled CCD camera and MetaMorph software.

Kinetics of Yap1-GFP Nucleocytoplasmic Localization

Exponentially growing yeast were exposed to hydrogen peroxide (400 μM) only or dimedone (50 mM) and then hydrogen peroxide (400 μM). Samples were fixed before and after peroxide treatment as described above. At each time point, cells (n = 200–300) were scored for subcellular localization of Yap1-GFP. Nuclear localization of Yap1-GFP was verified by colocalization with DAPI stain (Figure S2). Experiments were performed in duplicate, and data are presented as the average of the two trials and with corresponding standard deviations.

Immunoprecipitation of Gpx3-FLAG from *S. Cerevisiae*

Cells from the Yap1-GFP/Gpx3-FLAG strain (4×10^8 for in vitro studies, 1.25×10^8 for in vivo studies) were harvested, resuspended in lysis buffer (925 mM HEPES [pH 7.5], 35 mM NaCl, 2 mM EDTA, 1x yeast protease inhibitor cocktail, 40 μM chymostatin), and lysed by mechanical disruption using glass beads. Gpx3-FLAG was immunoprecipitated from lysate with EZView Red ANTI-FLAG M2 affinity gel (Sigma) for 1–4 hr at 4°C. The resin was collected at 8200 x *g* for 30 s and washed three times with 25 volumes of wash buffer (50 mM Tris HCl, 150 mM NaCl [pH 7.4]). Gpx3 was eluted with three volumes of elution buffer 1 (50 mM Tris HCl [pH 7.5], 150 mM NaCl, 1 mg ml^{-1} 1xFLAG peptide or 0.5 mg ml^{-1} 3xFLAG peptide).

Analysis of Gpx3 Intramolecular Disulfide Formation In Vitro

Recombinant wild-type Gpx3 was reduced with dithiothreitol (DTT) (50 mM) for 1.5 hr at rt. Reducing agent was removed by gel filtration using p-30 micro Bio-Spin columns (BioRad). Wild-type Gpx3 (60 μM) was then treated with dimedone (50 mM) or DMSO in the presence of hydrogen peroxide (50 μM) for 10 min at rt. Reactions were then incubated with iodoacetamide (300 mM) for 10 min at rt, resolved by nonreducing SDS-PAGE on 4%–12% Bis-Tris gels (Invitrogen), and visualized by Coomassie blue staining.

DAz-1 Labeling of Recombinant Gpx3 and Gpx3-FLAG

Recombinant wild-type, Cys36Ser, Cys82Ser, and Cys36Ser Cys64Ser Gpx3 were reduced with DTT (50 mM) for 1.5 hr at rt. Reducing agent was removed by gel filtration using p-30 micro Bio-Spin columns (BioRad). Wild-type Gpx3 and mutants (0–50 μM) were then treated with DAz-1 (10 mM) or DMSO in the presence of hydrogen peroxide (50 μM) for 15 min at 37°C. Small molecules were separated from the reaction by ultrafiltration with Amicon Ultra Filters (10 KD, Millipore). Reactions were then diluted with an equal volume of Buffer D and concentrated by ultrafiltration. Azide-modified Gpx3 was biotinylated and analyzed as described below. For Gpx3-FLAG, protein was immunoprecipitated from yeast lysate (100 μg total protein) and treated with DAz-1 (1 mM) or DMSO followed by the addition of hydrogen peroxide (0–25 μM). Reactions were incubated at rt for 1 hr. Alternatively, yeast lysate (100 μg) was exposed to hydrogen peroxide (10 μM) and incubated at rt for 10 min before the addition of DMSO or DAz-1 (1 mM). Gpx3-FLAG was then immunoprecipitated as described above. For in vivo labeling of Gpx3-FLAG, exponentially growing yeast were treated with DAz-1 (50 mM) for 20 min before peroxide treatment. Cells were exposed to hydrogen peroxide (400 μM) and grown at 30°C for 10 min. Cells were harvested and washed, and Gpx3 was enriched from the lysate (200 μg) as described above.

Biotinylation of Gpx3 and Western Blot Analysis

Azide-tagged Gpx3 was conjugated to biotin via Staudinger ligation with phosphine biotin (p-Biotin; 100–250 μM) for 2–4 hr at 37°C (Saxon and Bertozzi, 2000; Vocadlo et al., 2003). The resulting samples were subjected to SDS-PAGE and western blot analyses as previously described (Reddie et al., 2008) with the following modifications. Biotinylated proteins were detected by incubating polyvinyl difluoride (PVDF) membrane with 1:5,000–1:10,000 streptavidin-HRP (GE Healthcare) in Tris-buffered saline Tween-20 (TBST) or phosphate-buffered saline Tween-20 (PBST). For recombinant Gpx3 studies, 6xHis-Gpx3 was detected by incubating the PVDF membrane with 1:50,000–1:100,000 HisProbe-HRP (Pierce). For yeast studies, Gpx3-3xFLAG was detected by incubating the PVDF membrane with 1:2,000–1:10,000 Anti-FLAG M2 (Stratagene) in TBST, washed in TBST (2 × 10 min), and then incubated with 1:10,000-1:50,000 goat anti-mouse-HRP (Pierce). Western blots were developed with chemiluminescence (GE Healthcare ECL Plus Western Blot Detection System) and imaged on a Typhoon 9410 or by film.

Analysis of Yap1-Gpx3 Intermolecular Disulfide Formation In Vivo

Exponentially growing yeast carrying p416-TEF-Myc-Yap1 Cys303Ala were treated with dimedone (50 mM), hydrogen peroxide was added to yeast cultures (0–1 mM), and cells were grown at 30°C for 2 min. Cultures were lysed with TCA (20% v/v) and protein precipitates were resuspended in NEM buffer (100 mM Tris HCl [pH 8.0], 1 mM EDTA, 1% SDS, 150 mM NEM, 1x yeast protease inhibitor cocktail, 40 mM chymostatin). The pH was neutralized with sodium hydroxide and the alkylation reaction proceeded at rt for 15 min. For immunoprecipitation, the Yap1 Cys 303Ala-Gpx3-FLAG complex was isolated from cell extracts as described above. Proteins were then resolved by nonreducing or reducing SDS-PAGE using NuPAGE 4%–12% or 8% Bis-Tris gels (Invitrogen) in NuPAGE MES running buffer, transferred to PVDF membrane, and blocked with 5% bovine serum albumin (BSA) in PBST overnight at 4°C or 1 hr at rt. The membrane was washed in PBST (2 × 10 min) and the Myc epitope was detected by incubation with 1:1000 Anti-Myc monoclonal antibody (Covance) at 4°C overnight, and washed in PBST, followed by 1:10,000 goat anti-mouse-HRP (Pierce). Alternatively, the Myc epitope was detected by incubating the PVDF membrane with 1:1000 Anti-Yap1 polyclonal antibody (Santa Cruz Biotechnology) at 4°C overnight and washed in PBST, followed by 1:25,000 goat anti-rabbit-HRP (Calbiochem).

SUPPLEMENTAL DATA

Supplemental Data include five figures and Supplemental References and can be found with this article online at http://www.cell.com/chemistry-biology/supplemental/S1074-5521(09)00027-1.

ACKNOWLEDGMENTS

We thank the Life Sciences Institute, the Leukemia & Lymphoma Society (Special Fellows Award #3100-07), and the American Heart Association (Scientist Development Grant #0835419N to K.S.C.) for support of this work. We also thank Lois Weisman for the Yap1-GFP yeast strain, Daniel Klionsky for the *Δgpx3* yeast strain, Katrin Karbstein and Anuj Kumar for helpful discussions, and Thu H. Truong for her assistance with recombinant Gpx3 studies.

Received: August 27, 2008
Revised: January 14, 2009
Accepted: January 15, 2009
Published online: February 19, 2009

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