Molecular Mechanism of Oxidative Stress Perception by the Orp1 Protein*

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In this study we investigated the molecular mechanism by which the Orp1 (Gpx3) protein in Saccharomyces cerevisiae senses and reacts with hydrogen peroxide. Upon exposure to H$_2$O$_2$ Orp1Cys$_{36}$ forms a disulfide-bonded complex with the C-terminal domain of the Yap1 protein (Yap1-cCRD). We used 4-nitrobenzo-2-oxa-1,3-diazole to identify a cysteine sulfenic acid (Cys-SOH) modification that forms on Cys$_{36}^\text{Orp1}$ upon exposure to H$_2$O$_2$. Under similar conditions, neither Cys$_{82}^\text{Orp1}$ nor Cys$_{598}^\text{Yap1}$ forms Cys-SOH. A homology-based molecular model of Orp1 suggests that the structure of the active site of Orp1 is similar to that found in mammalian selenocysteine glutathione peroxidases. Proposed active site residues Gln$_{70}$ and Trp$_{125}$ form a catalytic triad with Cys$_{36}$ in the Orp1 molecular model. The remainder of the active site pocket is formed by Phe$_{38}$, Asn$_{126}$, and Phe$_{127}$, which are evolutionarily conserved residues. We made Q70A and W125A mutants and tested the ability of these mutants to form Cys-SOH in response to H$_2$O$_2$. Both mutants were unable to form Cys-SOH and did not form a H$_2$O$_2$-inducible disulfide-bonded complex with Yap1-cCRD. The p$_K_a$ of Cys$_{36}$ was determined to be 5.1, which is 3.2 pH units lower than that of a free cysteine (8.3). In contrast, Orp1 Cys$_{36}^{\text{Q70A}}$ (the resolving cysteine) has a p$_K_a$ value of 8.3. The p$_K_a$ of Cys$_{36}$ in the Q70A and W125A mutants is also 8.3, demonstrating the importance of these residues in modulating the nucleophilic character of Cys$_{36}$. Finally, we show that S. cerevisiae strains with ORP1 Q70A and W125A mutations are less tolerant to H$_2$O$_2$ than those containing wild-type ORP1. The results of our study suggest that attempts to identify novel redox-regulated proteins and signal transduction pathways should focus on characterization of low p$_K_a$ cysteines.

Oxidative stress has been defined as a cellular disturbance in the prooxidant-antioxidant balance in favor of prooxidants (1). Cellular antioxidant defense and detoxification pathways are formidable and allow organisms to limit the 10$^5$ oxidative DNA lesions that are estimated to form each day (2). An enormous body of evidence suggests that the production of reactive oxygen species (ROS) induces cellular damage and contributes to the etiology of degenerative diseases such as cancer (3–5). ROS are the main causes of oxidative stress and can include superoxide anion (O$_2^-$), hydrogen peroxide (H$_2$O$_2$), hydroxyl radical (HO$^*$), and alkyl hydroperoxides (ROOH). For example, exposure to the prevalent environmental toxicant arsenic induces oxidative stress in the form of increased levels of ROS (6, 7). If allowed to accumulate unchecked, these molecules exceed the normal antioxidant buffering capacity of the cell, leading to indiscriminate damage of cellular components, including DNA, proteins, and lipids (8–11).

Recent studies have demonstrated that ROS, such as hydrogen peroxides and alkyl hydroperoxides, can regulate signal transduction pathways, transcription factors, and gene expression in a variety of prokaryotic and eukaryotic organisms (12–19). Of particular relevance to this work are the common cysteine thiol switches that can regulate the biological function of proteins (20, 21). For example, the Escherichia coli transcription factor OxyR contains two conserved cysteine residues that are essential for oxidative perception and regulation (22). Upon exposure to H$_2$O$_2$, Cys$_{199}^{\text{OxyR}}$ of OxyR reacts with H$_2$O$_2$ to form a transient Cys-SOH intermediate (23). The Cys$_{199}^{\text{OxyR}}$ Cys-SOH immediately reacts with Cys$_{208}$ and forms a disulfide bond, which results in increased DNA binding affinity and OxyR target gene expression.

The budding yeast Saccharomyces cerevisiae is a valuable eukaryotic model for understanding the molecular and biochemical mechanisms involved in H$_2$O$_2$-regulated signal transduction pathways (24). The major H$_2$O$_2$ response pathway in S. cerevisiae involves the transcription factor Yap1 and the oxidant receptor protein Orp1 (12, 13, 25–27). The Yap1 oxidative stress response pathway is regulated by a novel disulfide bond-relay cascade and controls the expression of ~70 genes in response to H$_2$O$_2$ (28, 29). Orp1, which is homologous to glutathione peroxidase enzymes (hGpx), is responsible for H$_2$O$_2$ perception and subsequent catalysis of disulfide bond formation in Yap1 (12). The proposed peroxidase mechanism for Orp1 involves the initial H$_2$O$_2$ reaction with Cys$_{36}^{\text{Orp1}}$ (12). Based on the homology of Orp1 with selenocysteine containing hGpx enzymes, it has been proposed that Orp1 forms a sulfenic acid intermediate upon reaction with peroxides. Ultimately the Cys-SOH intermediate is resolved by Cys$_{36}^{\text{Orp1}}$ to form an intramolecular disulfide bond. This disulfide bond is then reduced via the thioredoxin pathway (12). For this reason, this class of non-selenocysteine hGpx-like enzymes has been classified as thioredoxin-dependent peroxidases or thiol peroxidases, with a similar function and mechanism to the peroxiredoxin family of proteins (30). The role of Orp1 in regulation of Yap1 involves the reaction of Cys$_{36}^{\text{Yap1}}$.
with Cys$^{36}$ resulting in a mixed disulfide bond complex. This complex has been captured in vivo through site-directed mutagenesis of the resolving Cys$^{303}$ residue in Yap1 (12). The mixed disulfide is then resolved by Cys$^{303}$ of Yap1 to form an intramolecular disulfide bond in Yap1. The Yap1 disulfide bond results in the masking of a nuclear export sequence in Yap1, nuclear accumulation of Yap1, and increased gene expression of Yap1 target genes (18, 28).

The molecular mechanism by which the Orp1 class of peroxiredoxins perceives hydrogen peroxide and alkyl hydroperoxide has not been characterized. In this study we demonstrate that upon reaction with H$_2$O$_2$, Orp1-Cys$^{36}$ forms a transient Cys-SOH on Cys$^{36}$. Our data suggest that the reactivity of Cys$^{36}$ is because of its extremely low pK$_a$ of 5.1. We also present a homology model of the Orp1 structure, which indicates that it has an active site structure similar to mammalian glutathione peroxidase. Mutation of the putative Gln$^{70}$, or Trp$^{125}$-active site residues to alanine abolishes H$_2$O$_2$-induced Cys$^{36}$ Cys-SOH formation and reverts the pK$_a$ of Cys$^{36}$ to 8.3.

**EXPERIMENTAL PROCEDURES**

**Chemicals**—7-Chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl), β-NADPH, iodoacetamide, thioreredoxin, and thioreredoxin reductase were purchased from Sigma. Dithiothreitol (DTT), H$_2$O$_2$, and 2-mercaptoethanol were purchased from Fisher. (2-Pyridyl)-dithiobimane (PDT-Bimane) was purchased from Toronto Research Biochemicals. Other chemicals were of the highest quality commercially available.

**Protein Cloning, Expression, and Purification**—The ORP1 gene was cloned from *S. cerevisiae* genomic DNA, subcloned into the pRSET vector, and purified as described previously (13). Orp1 single and double point mutants were made using standard PCR-based mutagenesis. The peroxidase activity was measured following established methodology (13). The Yap1-cCRD protein was designed and purified as described previously. Briefly, Cys$^{598}$ of Yap1 can then react with the Cys$^{36}$ SOH intermediate to form a mixed disulfide-bonded complex between Orp1 and Yap1 and ultimately results in the inhibition of a nuclear export sequence in Yap1 (18, 28). This complex has been identified with mass spectrometry in vivo (12). Additionally, a C310A/C315A/C620A/C629T mutant form of Yap1 has been shown to form disulfide bonds in vivo in a manner similar to wild-type Yap1 (12). These data indicated to us that a truncated Yap1 protein with only Cys$^{598}$ could serve as a useful tool in examining the interaction of Yap1 with H$_2$O$_2$-activated Orp1. After purification, all proteins were dialyzed into 25 mM Tris (pH 7.5), 100 mM NaCl, and 1 mM EDTA and stored at −80°C.

**Orp1 and Yap1-cCRD Interaction Assay**—Prior to use, Orp1 and Yap1-cCRD proteins were reduced with 50 mM DTT for 2 h at 25°C. After reduction, each protein was exchanged into 25 mM Tris buffer (pH 7.5), 100 mM NaCl, and 1 mM EDTA using a Hi-Trap desalting column (GE Healthcare) operating on an AKTA purifier fast protein liquid chromatograph. Proteins were concentrated to a final concentration of 40 μM with an Amicon Ultra-4 (10-kDa cutoff). 200-μl oxidation reactions were initiated with the addition of H$_2$O$_2$ followed immediately with NBD-Cl, to a final concentration of 80 and 250 μM, respectively. Reactions were incubated for 1 h at 25°C, and unreacted NBD-Cl was separated from protein with a 5-ml Hi-trap desalting column. NBD-Cl-labeled proteins were concentrated to 40 μM and analyzed with UV-visible spectroscopy on a Varian Cary 50 spectrophotometer.

**pK$_a$ Determination of Orp1 Sulfhydryls with PDT-Bimane**—The reaction of PDT-Bimane with cysteine forms pyridine-2-thione, which has a maximum absorption wavelength of 343 nm (32). This reaction has been used previously to determine the pK$_a$ value of cysteine residues in proteins (33). The reduced Orp1 proteins were diluted to 10 μM in sodium citrate or phosphate buffer ranging from pH 3.5 to 11.5. Reactions were started by addition of PDT-Bimane to a final concentration of 40 μM and rapidly mixed, and the absorbance at 343 nm was recorded over 120 min with a Varian Cary 50 Bio UV-visible spectrophotometer. The resulting 343 nm curves were fit to a single exponential ($Y = Y_0 + Ae^{-kt}$) function. The values for the inverse of the first-order rate constant ($t_1$) were plotted against pH. The resulting curves were fit to the Henderson-Hasselbach equation, and the pK$_a$ value was determined.

**Homology Model of Orp1**—The three-dimensional model of Orp1 was calculated based on the x-ray crystal structures of human Gpx2 and Gpx5. The structures of each protein have yet to be published but are deposited in the Protein Data Bank codes 2HE3 and 2I3Y, respectively. The model was constructed using MODELLER 9 version 1 following the detailed steps described in the MODELLER advanced tutorial (34, 35). Briefly, the primary sequences of the Gpx2 and Gpx5 templates were initially aligned using the align multiple structure/sequence alignment module within MODELLER. The Orp1 sequence was then aligned with the aligned sequences of hGpx2 and

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The Formation of Orp1Cys36 and Yap1-cCRD Disulfide Bond Complex—The goal of this study was to elucidate the molecular mechanism by which Orp1 senses peroxides and ultimately reacts with Yap1. Therefore, we developed a gel shift assay for monitoring the Orp1-Yap1 protein interaction. A Yap1-cCRD overexpression construct was created by amplifying the ORP1 coding region by PCR using oligonucleotides primers containing NotI (5′) and BglII (3′) sites. The PCR products were digested with NotI and BglII and ligated to ppp81 digested with NcoI and PstI (5′) sites. The resulting plasmids were verified by DNA sequencing. The wild-type and mutant ORP1 expression strains were created by transforming PacI linearized pADH1-ORP1 into the YMJW22 strain. As a control we also constructed a vector-only strain by transforming PacI linearized ppp81 into the YMJW22 strain. The integration of the ORP1 gene into the ADH1 locus was confirmed by colony PCR analysis. For H2O2 sensitivity analysis, each strain was grown to stationary phase (A600 = 0.7) in either YPD media or SC-URA minimal media. YPD plates were freshly prepared with 0, 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 mM H2O2 and were used immediately. Each strain was serially diluted, and 3 µl of each dilution was plated onto YPD plates. The plates were incubated at 30 °C for 2 days.

RESULTS

Identification of a Cys-SOH Intermediate on Cys36 of Orp1—To elucidate the molecular mechanism by which Orp1 senses H2O2, we wanted to determine whether the redox-active cysteines in Orp1 and Yap1-cCRD form Cys-SOH in response to H2O2. We used the chemical NBD-Cl to probe Cys-SOH modification of specific cysteines on Orp1 and Yap1-cCRD. NBD-Cl has been used previously to monitor Cys-SOH modification on proteins (31). One limitation of this technique is that to obtain unambiguous results, it requires the use of proteins that contain only one cysteine residue. Therefore, we constructed and purified two forms of Orp1 that contained either Cys36 or Cys82 and Yap1-cCRD that contained only Cys98. We first reacted Orp1Cys36 with H2O2 and NBD-Cl. As controls we prepared samples of Orp1Cys36 in parallel that were untreated and treated with NBD-Cl alone. The Orp1Cys36 and Yap1-cCRD were purified to homogeneity, reduced with DTT, and exchanged into buffer containing EDTA. It was observed that the presence of EDTA minimized protein oxidation, thus allowing all experiments to be performed aerobically. These assays were also conducted anaerobically and showed similar results (data not shown). Yap1-cCRD and Orp1Cys36 were mixed in equal molar ratios, and H2O2 was added to the reactions. Reactions were stopped with trichloroacetic acid after 1 min, separated with reducing or nonreducing SDS-PAGE, and stained with Coomassie Brilliant Blue (Fig. 1). The gel shows that Orp1Cys36 alone treated with H2O2 forms a small amount of disulfide-bonded dimer. This has also been observed with Orp1 homologs from poplar trees (37). Yap1-cCRD appears unchanged by H2O2 treatment and migrates at the same apparent molecular weight. However, mixtures of Orp1Cys36 and Yap1-cCRD form a higher molecular weight complex upon H2O2 treatment. This complex migrates at the appropriate molecular weight of an Orp1Cys36 and Yap1-cCRD disulfide bond-linked complex. The band corresponding to the Orp1Cys36, Yap1-cCRD complex is not observed in the individual Orp1Cys36 or Yap1-cCRD reactions. When these reactions are run under reducing conditions the Orp1Cys36, Yap1-cCRD complex is not observed, further demonstrating that Orp1Cys36 and Yap1-cCRD are covalently linked via a disulfide bond. These experiments provide evidence that Yap1-cCRD can specifically react with Orp1 in an H2O2-inducible manner, and we hypothesized that an intermediate of the reaction involved Cys-SOH formation on Orp1.

Analysis of Orp1 Mutations in Vivo—Yeast strains YPH499 (MATa ura3-52 lys2–801_amber ade2-101_ochre trp1-Δ63 his3-Δ200 leu2-Δ1) and YMJW22 described previously (YPH499Δorp1::KanMX4) were used and have been described previously (18). For Orp1 expression in S. cerevisiae, we used the ppp81 vector, which allows for stable integration of the plasmid DNA into the ADH1 promoter region (36). The pADH1-ORP1 overexpression constructs were created by amplifying the ORP1 coding region by PCR using oligonucleotides primers containing NotI (5′) and BglII (3′) sites. The PCR products were digested with NotI and BglII and ligated to ppp81 digested with NcoI (5′) and PstI (3′) sites. The resulting plasmids were verified by DNA sequencing. The wild-type and mutant ORP1 expression strains were created by transforming PacI linearized pADH1-ORP1 into the YMJW22 strain. As a control we also constructed a vector-only strain by transforming PacI linearized ppp81 into the YMJW22 strain. The integration of the ORP1 gene into the ADH1 locus was confirmed by colony PCR analysis. H2O2 sensitivity analysis, each strain was grown to stationary phase (A600 = 0.7) in either YPD media or SC-URA minimal media. YPD plates were freshly prepared with 0, 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 mM H2O2 and were used immediately. Each strain was serially diluted, and 3 µl of each dilution was plated onto YPD plates. The plates were incubated at 30 °C for 2 days.

FIGURE 1. In vitro analysis of Orp1Cys36 and Yap1-cCRD disulfide-linked complex formation. Formation of the Orp1Cys36-YP1-cCRD complex was performed aerobically at 25 °C (pH 7.5) with equimolar ratios of Orp1Cys36 and Yap1-cCRD and addition of H2O2. Each lane represents a reaction that was precipitated with trichloroacetic acid and separated with either reducing or nonreducing SDS-PAGE. Only when Orp1Cys36 and Yap1-cCRD are exposed to H2O2 is appreciable disulfide bond formation initiated. Under reducing conditions, the Orp1Cys36 and Yap1-cCRD disulfide-linked complex is not observed.

hGpx5 using the multiple alignment module. Five models of Orp1 were calculated using the model module and were analyzed with the discrete optimized protein energy function. We selected the lowest energy structure for modeling purposes and used MacPyMOL for displaying the structure.
Cys\textsuperscript{36} (R-SH) and oxidized (R-SOH) forms of the Orp1\textsuperscript{Cys\textsubscript{36}} give rise to distinctive spectra (A) with maximum absorption wavelength at 420 nm (R-S-NBD, dotted line) and 347 nm (R-S(O)-NBD, dashed line). The spectra of Orp1\textsuperscript{Cys\textsubscript{36}} itself is shown as a solid black line. Reacting to either Yap1-cCRD (B) or Orp1\textsuperscript{Cys\textsubscript{82}} (C) with H\textsubscript{2}O\textsubscript{2} and NBD-Cl does not produce a 347 nm (R-S(O)-NBD, dotted line) absorption peak. For each of these proteins only the 420 nm peak (R-S-NBD, dotted line) is observed, indicating that they do not form Cys-SOH. a.u., absorbance units.

![Figure 2](image)

**FIGURE 2. NBD adducts of cysteine thiols and sulfenic acids.** The reduced (R-SH) and oxidized (R-SOH) forms of the Orp1\textsuperscript{Cys\textsubscript{36}} give rise to distinctive spectra (A) with maximum absorption wavelength at 420 nm (R-S-NBD, dotted line) and 347 nm (R-S(O)-NBD, dashed line). The spectra of Orp1\textsuperscript{Cys\textsubscript{36}} itself is shown as a solid black line. Reacting to either Yap1-cCRD (B) or Orp1\textsuperscript{Cys\textsubscript{82}} (C) with H\textsubscript{2}O\textsubscript{2} and NBD-Cl does not produce a 347 nm (R-S(O)-NBD, dotted line) absorption peak. For each of these proteins only the 420 nm peak (R-S-NBD, dotted line) is observed, indicating that they do not form Cys-SOH. a.u., absorbance units.

Cys\textsuperscript{36} (R-SO-NBD). Both the 347 and 420 nm peaks disappeared upon reduction with DTT, indicating that the NBD-Cl molecule was connected via Cys\textsuperscript{36} (data not shown). We repeated these sets of experiments with both Orp1\textsuperscript{Cys\textsubscript{82}} and Yap1-cCRD (Fig. 2, B and C). In both cases the spectra of NBD-Cl-treated samples looked the same with absorbance peak maxima at 420 nm, regardless of whether H\textsubscript{2}O\textsubscript{2} was added or not. These data suggest that Orp1 reacts with H\textsubscript{2}O\textsubscript{2} at Cys\textsuperscript{36} and forms a Cys-SOH intermediate, but that neither Cys\textsuperscript{82} of Orp1 nor Cys\textsuperscript{598} of Yap1 forms Cys-SOH in the presence of H\textsubscript{2}O\textsubscript{2}.

**FIGURE 3. Molecular model of the Orp1 protein.** A, protein structure is shown as a ribbon diagram. The proposed active site residues Cys\textsuperscript{36}, Gln\textsuperscript{70}, and Trp\textsuperscript{125} are depicted by sticks with carbon, nitrogen, oxygen, and sulfur atoms colored green, red, blue, and orange, respectively. The side chain of the peroxidatic Cys\textsuperscript{36} is located ~10 Å below the Cys\textsuperscript{36}-active site. B, Orp1-active site formed by evolutionarily conserved residues. On the hydrophobic left side of the pocket are Phe\textsuperscript{38} and Phe\textsuperscript{127} residues. On the polar right side of the pocket are Gln\textsuperscript{70}, Trp\textsuperscript{125}, and Asn\textsuperscript{126}. The side chains of both Trp\textsuperscript{125} and Gln\textsuperscript{70} are within hydrogen bonding distance of the Cys\textsuperscript{36} sulfhydryl.

**Molecular Modeling of Orp1—**To better understand the molecular environment of Cys\textsuperscript{36} in the Orp1-active site, we constructed a homology model of the Orp1 protein. Previous studies have shown that proteins in the Orp1 family of peroxidases are thioredoxin-dependent and monomeric (12, 38). This differs from their human counterparts, which are glutathione-dependent, contain a seleno-cysteine in their active sites, and form tetramers in solution (39). We used the x-ray crystallographic structure of hGpx2 and hGpx5 as templates for constructing the Orp1 model. The overall fold of the lowest energy model of Orp1 is shown in Fig. 3A. The side chains of the Cys\textsuperscript{36}-active site and Cys\textsuperscript{82} are displayed. Also shown are the side chains of the conserved Gln\textsuperscript{70} and Trp\textsuperscript{125} residues, which are located around Cys\textsuperscript{36} in the active site. These residues are also located in the active site of human seleno-cysteine glutathione peroxidases. In the Orp1 model, Cys\textsuperscript{82} is located on the surface of the protein and is partially solvent-exposed. It is ~10 Å away...
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Analysis of the pKₐ of Orp1 Sulphydrys—We hypothesized that the sulphydryl pKₐ of Cys³⁶ is lowered by its molecular environment. To quantify the pKₐ of Orp1Cys³⁶ and Orp1Cys⁸², we used a thiol reactivity assay described previously by Brennan and co-workers (33) for measuring the pKₐ value of the active site cysteine in the OhrR transcription factor. Briefly, the chemical PDT-Bimane is reacted with a protein that contains a single cysteine residue over a range of pH values (32). The reaction is exponential function. We performed this analysis on the Orp1Cys³⁶ protein over a range of pH values from 3.5 to 8.5 (Fig. 5A). These data show that at pH values of 3.5 and 4.0, the reaction of Orp1Cys³⁶ with PDT-Bimane is slow and largely unchanged. This is what would be expected for a cysteine sulfhydryl that is protonated. Between the pH values of 4.5 and 6.0, the reaction of Orp1Cys³⁶ with PDT-Bimane is accelerated by a factor of 90-fold. At pH values 7.0 and above, the reaction is extremely fast and complete within 2 min. The t½ values were normalized and plotted as function of pH (Fig. 5B). These points were fit to the Henderson-Hasselberger Equations (34). The pKₐ of Cys³⁶ is 4.9 ± 0.1, in agreement with other reports (13).

Mutational Analysis of Orp1—To further examine the roles of Gln⁷⁰ and Trp¹²⁵ in the formation of Cys-SOH on Cys³⁶ upon H₂O₂ exposure, we made four versions of the Orp1 protein. To measure the hydrogen peroxidase activity of Orp1, we mutated Gln⁷⁰ and Trp¹²⁵ to alanine. To measure the ability of Orp1 to form a H₂O₂-inducible disulfide-bonded complex with Yap1-cCRD and Cys-SOH on Cys³⁶, we prepared Q⁷⁰A and W¹²⁵A mutants of Orp1 that only contained the Cys³⁶. We monitored the hydrogen peroxidase enzymatic activity of Orp1, Orp1Q⁷⁰A, and Orp1W¹²⁵A using a previously established NADPH-linked assay (13). This assay showed that Orp1Q⁷⁰A is no longer active as a peroxidase and that the Orp1W¹²⁵A mutant retained ~10% of wild-type peroxidase activity (Fig. 4A). Next we examined the ability of Orp1Cys³⁶/Q⁷⁰A and Orp1Cys³⁶/W¹²⁵A to form Cys-SOH in response to H₂O₂. NBD-Cl modification experiments were conducted as described previously with similar controls. Wild-type Orp1 formed Cys-SOH as judged by the absorbance peak at 347 nm (Fig. 4B). Both Orp1Cys³⁶/Q⁷⁰A and Orp1Cys³⁶/W¹²⁵A reacted with NBD-Cl in the absence of H₂O₂ and displayed an absorbance peak at 420 nm. Upon addition of H₂O₂ and NBD-Cl, neither Orp1Cys³⁶/Q⁷⁰A nor Orp1Cys³⁶/W¹²⁵A displayed an absorbance peak at 347 nm. These data suggest that Orp1Cys³⁶/Q⁷⁰A and Orp1Cys³⁶/W¹²⁵A are incapable of forming Cys-SOH in response to H₂O₂. Finally, we assayed the ability of Yap1-cCRD to react with Orp1Cys³⁶/Q⁷⁰A and Orp1Cys³⁶/W¹²⁵A to form a disulfide-bonded complex. We individually mixed Orp1Cys³⁶, Orp1Cys³⁶/Q⁷⁰A, Orp1Cys³⁶/W¹²⁵A, or Orp1Cys³⁶/H¹¹⁰A with Yap1-cCRD in equimolar ratios, added H₂O₂, and separated the reactions on reducing and nonreducing SDS-PAGE (Fig. 4C). Both Orp1Cys³⁶/Q⁷⁰A and Orp1Cys³⁶/W¹²⁵A were unable to form significant amounts of Orp1-Yap1-cCRD disulfide-bonded complex upon H₂O₂ addition. Taken together these results indicate that Gln⁷⁰ and Trp¹²⁵ influence the reactivity of Cys³⁶. Without these residues, Orp1 cannot react with H₂O₂ to form a Cys-SOH intermediate and is not able to form mixed disulfide bonds with Yap1.

from the Cys³⁶-active site and thus to form a disulfide bond with Cys³⁶ and the helix, and loop containing Cys⁸² would have to be flexible. The Gln⁷⁰ and Trp¹²⁵ side chains are located within 3.5 Å of the Cys³⁶ sulfhydryl and thus are strong candidates for modulating the reactivity of Cys³⁶ toward peroxide. In bovine Gpx the catalytic triad of Cys⁵², Gln⁹⁰, and Trp¹⁶⁵ is thought to stabilize the selenocysteine through hydrogen bonding with the imino group of the tryptophan residue and the amido group of the glutamine residue (40, 41). A detailed model of the Cys³⁶-active site is shown in Fig. 3B. From this model it is apparent that Cys³⁶ is located in a solvent-accessible active site pocket. The conserved Phe³⁸ and Phe¹²⁷ hydrophobic residues line one side of the active site, and conserved polar side chains that could form hydrogen bonds with Cys³⁶ line the other side of the active site.

Molecular Mechanism of Oxidative Stress Perception by Orp1—To further examine the roles of Gln⁷⁰ and Trp¹²⁵ in the formation of Cys-SOH on Cys³⁶ upon H₂O₂ exposure, we made four versions of the Orp1 protein. To measure the hydrogen peroxidase activity of Orp1, we mutated Gln⁷⁰ and Trp¹²⁵ to alanine. To measure the ability of Orp1 to form a H₂O₂-inducible disulfide-bonded complex with Yap1-cCRD and Cys-SOH on Cys³⁶, we prepared Q⁷⁰A and W¹²⁵A mutants of Orp1 that only contained the Cys³⁶. We monitored the hydrogen peroxidase enzymatic activity of Orp1, Orp1Q⁷⁰A, and Orp1W¹²⁵A using a previously established NADPH-linked assay (13). This assay showed that Orp1Q⁷⁰A is no longer active as a peroxidase and that the Orp1W¹²⁵A mutant retained ~10% of wild-type peroxidase activity (Fig. 4A). Next we examined the ability of Orp1Cys³⁶/Q⁷⁰A and Orp1Cys³⁶/W¹²⁵A to form Cys-SOH in response to H₂O₂. NBD-Cl modification experiments were conducted as described previously with similar controls. Wild-type Orp1 formed Cys-SOH as judged by the absorbance peak at 347 nm (Fig. 4B). Both Orp1Cys³⁶/Q⁷⁰A and Orp1Cys³⁶/W¹²⁵A reacted with NBD-Cl in the absence of H₂O₂ and displayed an absorbance peak at 420 nm. Upon addition of H₂O₂ and NBD-Cl, neither Orp1Cys³⁶/Q⁷⁰A nor Orp1Cys³⁶/W¹²⁵A displayed an absorbance peak at 347 nm. These data suggest that Orp1Cys³⁶/Q⁷⁰A and Orp1Cys³⁶/W¹²⁵A are incapable of forming Cys-SOH in response to H₂O₂. Finally, we assayed the ability of Yap1-cCRD to react with Orp1Cys³⁶/Q⁷⁰A and Orp1Cys³⁶/W¹²⁵A to form a disulfide-bonded complex. We individually mixed Orp1Cys³⁶, Orp1Cys³⁶/Q⁷⁰A, Orp1Cys³⁶/W¹²⁵A, or Orp1Cys³⁶/H¹¹⁰A with Yap1-cCRD in equimolar ratios, added H₂O₂, and separated the reactions on reducing and nonreducing SDS-PAGE (Fig. 4C). Both Orp1Cys³⁶/Q⁷⁰A and Orp1Cys³⁶/W¹²⁵A were unable to form significant amounts of Orp1-Yap1-cCRD disulfide-bonded complex upon H₂O₂ addition. Taken together these results indicate that Gln⁷⁰ and Trp¹²⁵ influence the reactivity of Cys³⁶. Without these residues, Orp1 cannot react with H₂O₂ to form a Cys-SOH intermediate and is not able to form mixed disulfide bonds with Yap1.

Analysis of the pKₐ of Orp1 Sulphydrys—We hypothesized that the sulphydryl pKₐ of Cys³⁶ is lowered by its molecular environment. To quantify the pKₐ of Orp1Cys³⁶ and Orp1Cys⁸², we used a thiol reactivity assay described previously by Brennan and co-workers (33) for measuring the pKₐ value of the active site cysteine in the OhrR transcription factor. Briefly, the chemical PDT-Bimane is reacted with a protein that contains a single cysteine residue over a range of pH values (32). The reaction is exponential function. We performed this analysis on the Orp1Cys³⁶ protein over a range of pH values from 3.5 to 8.5 (Fig. 5A). These data show that at pH values of 3.5 and 4.0, the reaction of Orp1Cys³⁶ with PDT-Bimane is slow and largely unchanged. This is what would be expected for a cysteine sulfhydryl that is protonated. Between the pH values of 4.5 and 6.0, the reaction is monitored at 343 nm as a function of time and fit to a first-order exponential function. We performed this analysis on the Orp1Cys³⁶ protein over a range of pH values from 3.5 to 8.5 (Fig. 5A). These data show that at pH values of 3.5 and 4.0, the reaction of Orp1Cys³⁶ with PDT-Bimane is slow and largely unchanged. This is what would be expected for a cysteine sulfhydryl that is protonated. Between the pH values of 4.5 and 6.0, the t½ values were normalized and plotted as function of pH (Fig. 5B). These points were fit to the Henderson-Hasselberger Equations (34). The pKₐ of Cys³⁶ is 4.9 ± 0.1, in agreement with other reports (13).
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![Graph A](image)

**FIGURE 5.** *pKₐ* determination of sulfhydryls with PDT-Bimane. A, reaction of Orp1Cys³⁶ with PDT-Bimane was monitored at 343 nm at pH values ranging from 3.5 to 8.5. The increase at 343 nm results from the release of pyridyl-2-thione from PDT-Bimane. Each curve was fit to a single exponential function. The *pKₐ* values are 3.2 pH units lower than the *pKₐ* of free cysteine. B, inverse of the rate constants (*t₁*) were normalized for the reactions of PDT-Bimane with Orp1Cys³⁶, Orp1Cys³⁶/Q70A, Orp1Cys³⁶/W¹²⁵A, and Orp1Cys⁸² and plotted as a function of pH. The results are fit to the Henderson-Hasselbalch equation. From these curve fits sulfhydryl *pKₐ* values of 5.1, 8.3, 8.3, and 8.3 were determined for the Orp1Cys³⁶, Orp1Cys³⁶/Q70A, Orp1Cys³⁶/W¹²⁵A and Orp1Cys⁸², respectively.

**DISCUSSION**

The discovery that disulfide bond formation in the Yap1 transcription factor is regulated by a peroxidase-like enzyme has led to the realization that peroxidases and peroxiredoxin enzymes are not only involved in ROS scavenging but also function as peroxide sensors in vivo. The Orp1 protein is a member of a thiol peroxidase family of enzymes that is homologous to human glutathione peroxidases, yet it is functionally similar to the peroxiredoxin protein family (12). These classes of proteins have evolved to use similar biochemical mechanisms to form mixed disulfide bonds with other proteins, thereby activating disulfide bond relay cascades and modulating the function of transcription factors such as Yap1 and Pap1 (12, 13, 17, 42).

The goal of this study was to investigate the molecular mechanism by which the Orp1 class of peroxiredoxin-like enzymes senses H₂O₂. We initially established a disulfide bond formation assay between Orp1 and a portion of the Yap1 protein that
has been shown to react with Orp1 in vivo. A disulfide-bonded complex was observed between Orp1 and Yap1-cCRD immediately following treatment with H₂O₂. We showed that a mutant form of Orp1, in which only the active site Cys³⁶ is present, forms a Cys-SOH intermediate upon H₂O₂ exposure using an NBD-Cl chemical modification assay. Cys-SOH intermediates have been shown to have extremely short half-lives and have proven difficult to identify in proteins. For this reason, the remaining nucleophilic cysteines need to be removed from the protein being studied. In the case of Orp1, Cys³⁶-SOH can be attacked by Cys⁸² of Orp1 to form an intramolecular disulfide bond or by Cys⁹⁹⁸ of Yap1 to form a mixed disulfide-bonded complex. We also examined the ability of Cys³⁶ of Orp1 or Cys⁵⁹⁸ of Yap1 to form Cys-SOH in response to H₂O₂. Our data showed that neither cysteine could form Cys-SOH as judged by NDB-Cl reactivity. These data provided support for the idea that Cys-SOH could be used as a reliable biochemical marker for Orp1 activity. It also suggests that H₂O₂-induced Cys-SOH formation may be a general characteristic of redox-active cysteine residues.

In this study we provide a comprehensive analysis of the correlation between cysteine sulfhydryl pKₐ, peroxidase activity, H₂O₂-induced Cys-SOH formation, and Orp1-active site structure. It had been shown previously that the selenocyte of the human glutathione peroxidase enzymes reacts with H₂O₂ to form a selenol intermediate prior to reaction with glutathione (40). In addition, the seleno-cysteine-active site is formed by a catalytic triad involving Gln⁵³ and Trp¹⁵⁷. Mutation of either of these amino acids was shown to decrease the enzymatic activity of hGpx and reduced the ability of iodoacetic acid to inhibit hGpx activity (41). Our homology model of Orp1 showed that the homologous Gln⁷⁰ and Trp¹²⁵ were part of an active site pocket formed around Cys³⁶ (Fig. 3, A and B). One side of the pocket was entirely hydrophobic, lined with the conserved phenylalanine residues Phe³⁸ and Phe¹²⁷. These residues have van der Waals interactions with Cys³⁶ and appear to effectively shield Cys³⁶ from the bulk solvent. On the other side of the pocket are the side chains of Gln⁷⁰, Trp¹²⁵, and Asn¹²⁶, which are all conserved residues containing side chains that can make hydrogen bonds with the sulfhydryl-active site. Our results strongly suggest that the role of Gln⁷⁰ and Trp¹²⁵ in Orp1 reactivity is to stabilize the thiolate form of Cys³⁶. In the absence of H₂O₂, mutation of either residue abolishes the low pKₐ of Cys³⁶. Although the Cys³⁶-active site pocket is accessible to solvent, this pocket does not appear to be able to accommodate Cys³⁶-SOH. Therefore, upon oxidation the Cys-SOH would protrude from the active site pocket into the solvent, making it more vulnerable to nucleophilic attack by another cysteine.

The global identification of redox-active cysteine residues in proteins has been the focus of many recent investigations (43–45). Gladyshev and co-workers (44) used a novel bioinformatics approach to identify redox-active cysteines in the proteomes of multiple organisms. Their approach identified many previously characterized oxidoreductase proteins, such as the glutathione peroxidase family, which contain a nucleophilic active site cysteine. Proteins that contain cysteines with abnormally low pKₐ values have historically been characterized by their increased reactivity with haloacetic acids. Use of haloacetic acid modification in conjunction with monitoring enzymatic activity is a well-established approach for measuring cysteine pKₐ (46, 47). One example is Cys²⁵ in the sulfhydryl protease papain that has a pKₐ of ~4 (47). Our results and prior studies on nucleophilic active site cysteine suggest that one common biochemical feature of these active site cysteines is a lowered sulfhydryl pKₐ. In the case of Orp1, we determined the pKₐ of Cys³⁶ to be ~3.2 pH units lower than that of free cysteine. It is this feature alone that contributes to the reactivity of Orp1 toward peroxides and enables it to form Cys-SOH and, eventually, disulfide bonds.

There appear to be multiple ways that enzymes and transcription factors have evolved lower pKₐ cysteines, thereby increasing their reactivity toward peroxides. In the case of the OxyR transcription factor, the reactivity of Cys¹⁹⁹ appears to be modulated via an ion pair with Arg²⁶⁶. The x-ray crystal structure of OxyR shows that the side chain of Arg²⁶⁶ is buried in the hydrophobic core behind the Cys¹⁹⁹-active site (48). Although the pKₐ of Cys¹⁹⁹ has not been determined, it has been shown that Cys¹⁹⁹ forms Cys-SOH in response to H₂O₂ (23). The active site composition of OxyR suggests that the positively charged and buried Arg²⁶⁶ side chain stabilizes Cys¹⁹⁹ in the thiolate form. Two additional examples of redox-active cysteines that use ion pairing to lower their pKₐ can be seen in ArsC, an arsene reductase found in E. coli, and in AhpC, a peroxiredoxin found in E. coli (49). In the case of ArsC, the pKₐ of Cys¹² in its active site is 6.4 and is stabilized by the positive charge on His⁸. Mutation of His⁸ to glycine resulted in a decrease in the reactivity of the Cys¹² pKₐ back to that of free cysteine. Finally, an example of a low pKₐ cysteine that is neither part of a catalytic triad nor an ion pair can be found in the OhrR transcription factor from Bacillus subtilis (50). When reduced, OhrR can cooperatively bind its promoter and repress transcription (33). Upon reaction with organic hydroperoxides, OhrR is oxidized at Cys¹⁵ and forms a Cys-SOH. Using similar pKₐ determination techniques as those used in the present study, Brennan and co-workers (33) showed that OhrR has a pKₐ value of 5.2. The x-ray crystal structure of reduced OhrR shows that Cys¹⁵ sits at the end of an α-helix and is adjacent to two tyrosine residues. Brennan and co-workers (33) suggest that the peroxiredoxin found in E. coli (49). In the case of ArsC, the pKₐ of Cys¹² in its active site is 6.4 and is stabilized by the positive charge on His⁸. Mutation of His⁸ to glycine resulted in a decrease in the reactivity of the Cys¹² pKₐ back to that of free cysteine. Finally, an example of a low pKₐ cysteine that is neither part of a catalytic triad nor an ion pair can be found in the OhrR transcription factor from Bacillus subtilis (50). When reduced, OhrR can cooperatively bind its promoter and repress transcription (33). Upon reaction with organic hydroperoxides, OhrR is oxidized at Cys¹⁵ and forms a Cys-SOH. Using similar pKₐ determination techniques as those used in the present study, Brennan and co-workers (33) showed that OhrR has a pKₐ value of 5.2. The x-ray crystal structure of reduced OhrR shows that Cys¹⁵ sits at the end of an α-helix and is adjacent to two tyrosine residues. Brennan and co-workers (33) suggest that the positive macrdipole of the α-helix could explain the lower pKₐ value of Cys¹⁵, but biochemical studies to confirm this hypothesis have not been performed. What remains a major challenge in the field of protein redox regulation is the identification of proteins with low pKₐ cysteines. Identification of these cysteines will undoubtedly lead to the discovery of novel proteins that are regulated by thiol oxidation and reduction as well as other enzymes that utilize nucleophilic sulfhydrys as part of their catalytic mechanism.

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