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The role of active site residues in the oxidant specificity of the Orp1 thiol peroxidase ☆

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ABSTRACT

In this study we investigated the role of active site residues in the peroxidase activity of Orp1 (GPx3) using three different peroxide substrates. Using a structural homology model of the reduced form of Orp1, we identified Asn126 and Phe127 as evolutionarily conserved residues that line the back of the Orp1 active site and which are likely to affect the peroxidase activity of Orp1. Additionally, we identified Phe38 as a surface residue that could influence substrate specificity as it is located adjacent to Cys36, in the same position occupied by similar hydrophobic amino acids in many Orp1 homologs. We individually mutated these residues to alanine and examined the effect of each mutation *in vitro* and *in vivo*. Chloro-4-nitrobenzo-2-oxa-1,3-diazole was used to identify Cys-SOH modification of Cys36 in response to H_2O_2, *tert*-butyl-hydroperoxide (*tert*-BHP), and cumene hydroperoxide (CHP) in Orp1^{WT}. Mutation of Asn126 and Phe127 eliminate Cys-SOH formation and peroxidase activity in response to H_2O_2, *tert*-BHP and CHP. Furthermore, the pK_a of Cys36 is elevated closer to that of free cysteine compared to Orp1^{WT}. Mutation of Phe38 does not affect the peroxidase activity of Orp1 upon exposure to H_2O_2. The Phe38 mutation decreases Orp1 peroxidase activities in response to either *tert*-BHP or CHP. The *in vivo* sensitivity of the Phe38 mutant to both *tert*-BHP and CHP is increased, while the H_2O_2 sensitivity is unchanged. The pK_a of Cys36 in the Phe38 mutant is 5.0, which is the same as Orp1^{WT}. Taken together, these results suggest that Phe38 does not play a role in the reactivity of Cys36, but does modulate the affinity of Orp1 for alkyl hydroperoxides.

1. Introduction

The damage caused by reactive oxidative species (ROS) has been linked to a wide variety of human diseases such as atherosclerosis, Parkinson's disease, Alzheimer's disease, and cancer [1,2]. Therefore, the ability to sense and respond to a wide range of ROS is vital for the survival of all aerobic organisms. Intracellular ROS, such as hydrogen peroxide (H_2O_2) and alkyl hydroperoxides, occur naturally within cells, but also result from exposure to environmental contaminants such as cadmium and arsenic [3–6]. To counteract the damaging effects of ROS, the intracellular environment of the cell is maintained under reducing conditions in part by proteins that detoxify reactive oxidative species. Many of these proteins are involved in peroxide scavenging as well as the regulation of peroxide mediated signaling through complex redox-relay systems that have yet to be fully characterized [7].

Glutathione peroxidases (GPxs) form a large, phylogenetically conserved family of over 700 enzymes that utilize a peroxidatic cysteine (C_P) or selenocysteine (U_P) to reduce and thereby detoxify peroxides [8,9]. The three-dimensional environment surrounding C_P or U_P affect both its reactivity and accessibility. The Orp1 thiol peroxidase is homologous to glutathione peroxidases and plays a dual role in the oxidative stress response pathway in *S. cerevisiae*. It detoxifies both H_2O_2 and alkyl hydroperoxides, such as *tert*-butyl-hydroperoxide (*tert*-BHP) and cumene hydroperoxide (CHP), and mediates the oxidative stress response in concert with the Yap1 transcription factor [10,11]. The C_P (Cys36) on Orp1 has been shown to form a cysteine sulfenic acid (Cys-SOH) intermediate upon exposure to H_2O_2 [12]. The Cys-SOH intermediate either forms an intramolecular disulfide bond with resolving cysteine (Cys82), which is reduced by thioredoxin, or an intermolecular disulfide bond with Cys598 on Yap1, leading to its activation [10,11]. In Orp1, the residues Gln70 and Trp125 form the catalytic triad with Cys36. Mutation of Gln70 or Trp125 to alanine removes the ability of Cys36 to form Cys-SOH and increases the pK_a of Cys36 from 5.1 to 8.3 [12]. This work investigates the role of residues surrounding the Orp1 active site in moderating its peroxide reactivity.

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2. Materials and methods

2.1. Chemicals

Chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl), β-NADPH, *tert*-BHP and iodoacetamide, were purchased from Sigma–Aldrich. Thioredoxin reductase (*Escherichia coli* recombinant, lyophilized powder) was purchased from CalBiochem. Dithiothreitol (DTT), H_2O_2, and cumene hydroperoxide (CHP) were purchased from Fisher. (2-pyridyl)-dithiobimane (PDT-Bimane) was purchased from Toronto Research Biochemicals.

2.2. Homology model of Orp1

The three-dimensional model of reduced Orp1 was calculated based on the X-ray crystal structures of human hGPx2 (PDB code 2HE3) and hGPx5 (PBD code 2I3Y) as previously described [12].

2.3. 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 [12,13]. Orp1 single and double point mutants were made using standard PCR-based mutagenesis. The Yap1-cCRD protein was designed and purified as described previously [14].

2.4. Homology model of Orp1, detection of cysteine sulfenic acid modifications with NBD-Cl, pK_a determination of Orp1 sulfhydryls with PDT-Bimane

Experiments were carried out as previously described [12].

2.5. Enzyme activity assay

The decrease in absorbance of NADPH was used to indirectly measure peroxidase activity of Orp1 *in vitro*. 1.35 μM of purified wild-type Orp1 or mutant Orp1 was mixed with 1.35 μM TrxA, 1.44 μM TrxB, and 300 μM β-NADPH in a total volume of 100 μL in a buffer containing 100 mM Tris (pH 8.0) and 10 mM EDTA and incubated at 25 °C for 10 min. One hundred μM of peroxide (H_2O_2, CHP, or *tert*-BHP) in 1 μL was added to start the reaction and the absorbance of NADPH at 340 nm was measured every 15 s for 5 min. The results were baseline corrected and normalized using the absorbance from a reaction containing all components except peroxide.

2.6. Analysis of Orp1 mutations in vivo

In vivo analysis was carried out as previously described [12]. Yeast were normalized for cell number and serial dilutions were spotted onto freshly prepared YPD plates with 0, 0.75, 1.5, and 2.25 mM H_2O_2, 0, 0.05, 0.10, and 0.15 mM CHP, or 0, 0.15, 0.30, 0.45, and 0.60 mM *tert*-BHP and incubated at 30 °C for 2 days.

3. Results

3.1. Identification of Orp1 active site residues that modulate redox activity

The amino acid sequence of Orp1 is highly conserved among other members of the GPx family. Fig. 1A presents a protein sequence alignment of seven homologous proteins. Orp1 Cys36 is conserved in all aligned proteins. We hypothesized that conserved residues in structural proximity to Cys36 influence the peroxidase activity of Orp1 and would therefore have the greatest effect when mutated. To identify candidate residues, we used a homology model of reduced Orp1 based on the X-ray crystallographic structures of hGPx2 and hGPx5 (Fig. 1B). We identified Phe38, Asn126 and Phe127 as residues that would likely play a role in Orp1 redox activity. Phe38 and Phe127 are located within 4.20 and 3.07 Å, respectively, of Cys36, while the carboxamide group of Asn126 is located 4.47 Å away from Cys36. Both Asn126 and Phe127 are strictly conserved in Orp1 homologs (Fig. 1A). Conversely, Phe38 is conserved in only three of the seven Orp1 homologs. In the remaining three, this position is occupied by a leucine. The proximity to Cys36, coupled with its hydrophobicity and position on the surface of Orp1, indicate that Phe38 might play a role in Orp1 reactivity with peroxides such as *tert*-BHP and CHP.

3.2. Mutational analysis of Orp1 peroxidase activity

To determine if Phe38, Asn126 and Phe127 are required for the peroxidase activity of Orp1, we purified wild-type Orp1 and Orp1 containing point mutations of Phe38 to Ala ($Orp1^{F38A}$), Asn126 to Ala ($Orp1^{N126A}$), and Phe127 to Ala ($Orp1^{F127A}$) for use in peroxidase activity assays. For these assays, enzyme activity was measured using a spectrophotometric assay that indirectly measures the ability of Orp1 to reduce peroxides via a decrease in absorbance of NADPH at 340 nm [15]. Orp1 was mixed with *b*TrxA, *b*TrxB, and NADPH and the reaction was initiated with the addition of 100 μM H_2O_2, *tert*-BHP, or CHP. The rate of enzyme activity, was similar for wild-type Orp1 upon exposure to H_2O_2 or *tert*-BHP, but was decreased for CHP (Fig. 2A). $Orp1^{F38A}$ showed slightly decreased peroxidase activity compared to WT Orp1 upon addition of *tert*-BHP, and CHP (Fig. 2B). In contrast, both $Orp1^{N126A}$ and $Orp1^{F127A}$ showed a dramatic loss of peroxidase activity for all three peroxides (Fig. 2C and D).

3.3. Characterization of Cys36 sulfenic acid formation in response to peroxides

To further characterize the $Orp1^{F38A}$, $Orp1^{N126A}$ and $Orp1^{F127A}$ mutations, we monitored the formation of Cys-SOH on Cys36. We used the chemical 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl) to probe for Cys-SOH modification of Cys36 in response to upon addition of H_2O_2, *tert*-BHP, and CHP [15]. We have previously shown that the Orp1 active site Cys36 forms Cys-SOH in response to H_2O_2 [12]. To determine if the mutation of residues Phe38, Asn126, or Phe127 affect the formation of Cys-SOH on Cys36, we used a previously characterized Orp1 mutant in which Cys64 and Cys82 were mutated to serine ($Orp1^{C36}$). We subsequently mutated Phe38 to Ala ($Orp1^{C36/F38A}$), Asn126 to Ala ($Orp1^{C36/N126A}$), and Phe127 to Ala ($Orp1^{C36/F127A}$).

We treated $Orp1^{C36}$ with H_2O_2, *tert*-BHP, or CHP before the addition of NBD-Cl. As a negative control $Orp1^{C36}$ was reacted with NBD-Cl alone. Unreacted NBD-Cl was removed from all solutions by gel filtration chromatography and protein samples were concentrated prior to UV–visible spectroscopy. Fig. 3A shows the spectra of $Orp1^{C36}$. The $Orp1^{C36}$ protein alone shows a peak with a maximal absorbance at 280 nm, while the spectrum of $Orp1^{C36}$ combined with NBD-Cl shows an additional peak with a maximal absorbance at 420 nm. The presence of a peak at 420 nm is indicative of the reaction of NBD-Cl with the sulfhydryl form of Cys36 (R-S-NBD). The reaction of NBD-Cl and $Orp1^{C36}$ in the presence of H_2O_2, *tert*-BHP, or CHP all show peaks with maximal absorbance at 347 nm (Fig. 3A). The presence of the 347 nm peak is indicative of Cys-SOH formation on Cys36 (R-SO-NBD). The results for *tert*-BHP and CHP are consistent with previous results obtained with $Orp1^{C36}$. The reaction of NBD-Cl with $Orp1^{C36/F38A}$ after addition of H_2O_2 results in a similar spectrum as that of $Orp1^{C36}$ (Fig. 3B). This spectrum