Biochemical Characterization of 2-Cys Peroxiredoxins from
Schistosoma mansoni

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Ahmed A. Sayed and David L. Williams‡
From the Department of Biological Sciences, Illinois State University, Normal, Illinois 61790-4120

Peroxiredoxins are a large family of peroxidases that have important antioxidant and cell signaling functions. Genes encoding two novel 2-cysteine peroxiredoxin proteins were identified in the expressed sequence tag data base of the helminth parasite Schistosoma mansoni, a causative agent of schistosomiasis. The recombinant proteins showed peroxidase activity in vitro with a variety of hydroperoxides and used both the thioredoxin and the glutathione systems as electron donors. Steady-state kinetic analysis indicated that the new peroxiredoxins had saturable kinetics, whereas a previously identified schistosome peroxiredoxin was found to function with more typical unsaturable (ping-pong) kinetics. The catalytic efficiencies S. mansoni peroxiredoxins were similar to those for other peroxiredoxins studied ($10^3–10^5$ M$^{-1}$ s$^{-1}$). Mutagenesis of S. mansoni peroxiredoxins indicated that glutathione dependence and kinetic differences were conferred by the C-terminal o-helix forming 22 amino acids. This is the first report of 2-cysteine peroxiredoxins efficiently utilizing reducing equivalents from both the thioredoxin and glutathione systems. Studies to determine the resistance to oxidative inactivation, important in regulating cell signaling pathways, showed that S. mansoni possess both bacterial-like resistant and mammalian-like sensitive peroxiredoxins. The susceptibility to oxidative inactivation was conferred by the C-terminal tail containing a tyrosine-phenylalanine motif. S. mansoni is the first organism shown to possess both robust and sensitive peroxiredoxins. The ability of schistosome peroxiredoxins to use alternative electron donors, and their variable resistance to overoxidation may reflect their presence in different cellular sites and emphasizes the significant differences in overall redox balance mechanisms between the parasite and its mammalian host.

Schistosoma mansoni, a trematode worm, is a causative agent of schistosomiasis, a severe and debilitating disease affecting ~250 million people in the tropics and resulting in ~280,000 deaths annually (1, 2). Schistosomes reside in the bloodstream of their host surviving many years (5–30 years). One proposed survival mechanism is the production of protective antioxidant proteins to neutralize oxidative damage resulting from the host’s immune response as well as self-generated oxygen radicals (3). Schistosomes lack catalase (4), the main H$_2$O$_2$-neutralizing enzyme of many organisms, and their glutathione peroxidases (GPx) are in the phospholipid hydroperoxide GPx class (GPx4) with poor reactivity toward H$_2$O$_2$ (5, 6). With high levels of superoxide dismutase (4, 7) and in the absence of catalase, GPx may not effectively control H$_2$O$_2$ levels in the worm. Therefore, we sought to identify additional schistosome antioxidant enzymes that would neutralize H$_2$O$_2$. Potentially the identification and characterization of these enzymes will contribute to an anti-schistosome vaccine or chemotherapy targeting the redox system of the parasite.

Peroxiredoxins (Prxs) are a novel class of antioxidants recently described in a wide variety of organisms (8–11). Evidence suggests that Prxs are involved in redox balance and redox signaling and affect protein phosphorylation, transcriptional regulation, and apoptosis in animals (11). In our previous work we described a Prx (referred to as thioredoxin peroxidase) in schistosomes with biochemical properties similar to known Prx enzymes using thioredoxin as an electron donor (12). This enzyme was reasonably abundant in all stages tested and was found at elevated levels in the parasite egg and egg secretions (12, 13).

During its catalytic cycle Prx becomes oxidized through the formation of sulfenic acid derivatives of cysteine (8, 14, 15). Reduction of these intermediates is accomplished by thioredoxin (Trx) in most eukaryotic cells. Thioredoxin itself is maintained in the reduced state by Trx reductase (TrxR), an NADPH-dependent pyridine nucleotide-disulfide oxidoreductase (16). A parallel thiol-based redox system utilizing GPs, glutathione (GSH), and GSH reductase (GR) exists in most cells (17). The thiol-based reducing system in S. mansoni has been shown to have unique properties. Schistosomes lack both TrxR and GR and instead have an unusual pyridine nucleotide-disulfide oxidoreductase Trx-GSH reductase (TGR) functioning on both Trx and GSH reduction (18).

In this context, thiol-dependent redox mechanisms in schistosomes appear intriguing. Therefore, in this study we sought to further our understanding of thiol-dependent hydroperoxide metabolism in S. mansoni. Two novel schistosome Prx enzymes were identified, recombinantly expressed in Escherichia coli, and biochemically characterized. As expected, the new proteins were active with Trx on a range of hydroperoxide substrates. Additionally, these new proteins showed an unusual ability to utilize GSH as reducing agent. Furthermore, GSH affinity was shown to be conferred by the 22 C-terminal amino acids. The new proteins also were shown to be sensitive to overoxidation by hydroperoxides; the previously characterized Prx was found
to be remarkably resistant to overoxidation. The evolution of Prx enzymes in schistosomes as GSH-utilizing and -sensitive-resistant isoforms shows the importance of these enzymes in the thiol-dependent hydroperoxide metabolism of this important human parasite.

EXPERIMENTAL PROCEDURES

Identification of Novel S. mansoni Peroxiredoxins—To identify novel Prx genes, the GenBankTM EST data base was queried by tblastn with the open reading frame of Prx1 (12). A novel Prx sequence (Prx2) was identified in EST M EcTrx and M EcTrxR, which was obtained from Dr. Jucara Para, Belo Horizonte, Brazil and sequenced in its entirety. The complete sequence was deposited in GenBankTM with the accession number AF157561. A second novel Prx sequence (Prx3) was identified in the EST data base and cloned by PCR from a mixed-sex adult cDNA library. The 5′-end was cloned using a primer (5′-CACACCCGGGTTCG-GAGACG-3′) designed from EST AW017130 and the T3 promoter primer of pScript. The PCR product was cloned and sequenced, and a primer was designed (5′-CCTGGTCTACCTGTGCAGAGA-3′) to amplify the 3′-end of the gene from the cDNA library with the T7 promoter primer of pScript. The complete cDNA sequence of Prx3 was deposited in GenBankTM with the accession number AF301001. Analysis of S. mansoni encoded Prx proteins was by ClustalW (version 1.7/19). Isolation from the S1 baculovirus and sequencing of S. mansoni Prx genes was as described previously (10).

Recombinant Protein Production and Purification—The entire open reading frame of each S. mansoni Prx was amplified by PCR (21) using oligonucleotide primers modified to contain BamH1 and HindIII restriction sites to subclone subcloning after insertion into plRSETA expression plasmid (12) and TsgDNA polymerase (Promega, Madison, WI). Primers used were Prx2F (5′-CTGAACTTCTTACGAGGAAAGA-3′), Prx2R (5′-CTCGAGTTATGCTGCTATTATGAGA-3′), Prx3F (5′-CTGAGATCGTTATGCTGCTATTATGAGA-3′), and Prx3R (5′-CTGAGATCGTTATGCTGCTATTATGAGA-3′). The 33 N-terminal amino acids constituting the mitochondrial-targeting sequence of Prx3 were not included in the expressed plasmid. Open reading frames in plRSETA were verified by nucleotide sequencing. The induction of recombinant proteins in E. coli strain BLR(DE3)/pLysS (Novagen, Madison, WI) was performed as described (12). In the case of Prx2 and Prx3 cells were sonicated in 5% monothioglycerol (3-mercaptopropanediol) and 10 mM imidazole in start buffer (0.07 mM NaHPO4, 0.01 mM NaH2PO4, and 0.15 mM NaCl, pH 7.4). The supernatant was filtered and applied to pre-equilibrated His-Trap column (Amersham Biosciences). The desired protein was then eluted from the column by using increasing concentrations of imidazole 5% monothioglycerol in start buffer. Protein concentration was then checked by Bio-Rad protein assay. Protein purity was checked by using SDS-PAGE. The purified proteins were dialyzed against phosphate-buffered saline and stored at −20° C until used.

Expression and Purification of Recombinant E. coli Thioredoxin and Thioredoxin Reductase—The coding sequences for E. coli (Ec)Trx and EcTrxR were cloned into plRSETA as described above. Flavin mononucleotide was added to sonicates of cells expressing EcTrx and EcTrxR by consumption of NADPH. The induction of EcTrxR was performed by cloning. C-truncated Prx2 protein was used with Prx2 to amplify the complementary strand in PCR2. The reverse mutated Prx2 primer ligated into plRSETA-expressing vector served as template for PCR clone. The forward mutated primer (underlined) 5′-CAT GGC GAA GTT TCT CCA GGC AAC-3′ was used with Prx2F to amplify the first mutated strand in PCR1. The reverse mutated Prx2 primer 5′-CCA GTC GAA TCC GCC CTC-3′ was used with Prx2F to amplify the complementary strand in PCR2. The two primary PCR products were then mixed equally, denatured, and allowed to re-anneal at 40 °C. The overlapping (21 nucleotides) heteroduplexes with recessed 3′-ends were extended by 40 cycles at (96 °C for 0 s, 50 °C for 0 s, and 72 °C for 5 min) in PCR2. The extended product from PCR3 was annealed with Prx3F and Prx3R primers in PCR4. The product from PCR4 was cloned into plRSETA vector using TOPO TA cloning (Invitrogen). The sequence of mutated Prx2 in which Cys174 is replaced by serine (C168S) was verified by sequencing. The Prx2 C168S expression plasmid was generated by digestion the PCRII vector with BamH1 and HindIII, separating the desired fragment on low melting agarose gel, using β-agarase (New England Biolabs, Beverly, MA) digestion for 1 h at 40 °C, purified with phenol/chloroform extraction, and ligated into predigested and purified pRSETA expression vector with the Quick Ligate kit (New England Biolabs). The sequence was verified again by sequencing before transformation into chemically competent (21) BLR(DE3)/pLysS cells. Induction and purification of recombinant protein was performed as described above.

Tailed S. mansoni Prx1 Cloning and Overexpression—The 12 C-terminal amino acids (19KRGHGVKQNRK26) of Prx1 were replaced with the 22 C-terminal amino acids of Prx2 (21KPNASITIKDPVASYL- SYFSSVHY27) using PCR reaction with specifically designed primers. A 91-nucleotide nucleotide reverse primer, including the C-terminus of Prx2 plus an 18-amino acid overlapping sequence with Prx1 (5′-GGCA AGG CTT TCA GTC CAC AGA GGA GAA GTA GGA GAG AGA AAC AGG ATC AGG TAT TGT TGT TGC TGA ATT TGG TTG CTC GAT CGG ACA-3′) was used with Prx1F (5′-CAGC-GATCCCCATGTTAGCTTGGCATA-3′). The tailed Prx1 was then cloned into plRSETA using a TOPO TA cloning kit. The sequence of the tailed Prx1 insert was verified. Recombinant tailed SmPrx1 was generated in a pRSETA expression system and purified as described above. C-terminally Truncated SmPrx2—The 21 amino acid C-terminally (C-) truncated Prx2 was amplified by polymerase chain primers Prx2F and Prx2RM 5′-GGCAACGTTCATTTTATTCTGCTTGGCTT-3′ and 5′-TCGATCCGACCGGACCGACC-3′ to amplify the 3′-end of the gene from the cDNA library with the T7 promoter primer of pScript. The complete cDNA sequence of Prx2 was deposited in GenBankTM with the accession number AF157561. A second novel Prx sequence (Prx3) was identified by tblastn search of the GenBankTM EST data base identified two novel 2-cysteine Prx genes as described above (10). A novel Prx sequence (Prx2) was amplified with the open reading frame of Prx1, Prx2, C-truncated Prx2, and tailed-Prx1 was calculated by percentage of the initial enzyme rate. The rate of peroxide inactivation was expressed as units per milligram of protein (as determined by Bio-Rad protein assay) (25). Background NADPH consumption was measured in the absence of peroxidoxins. Enzymatic oxidation of NADPH was initiated by adding hydroperoxide (0.5 mM) to the sample cuvette and monitoring A340 for 2 min. Background rates were subtracted from the experimental rates. The specific activity of the sample was determined from the equation, units of Prx/mg of protein = [(Vmax − Vo) × 6.622 × 1000]/([protein] (in milligrams/ml), where 6.622 = extinction coefficient of NADPH (mM cm−1), Vmax is the total reaction volume, V0 is the sample volume, and 1000 is used to convert to nanomoles. One unit of Prx activity is defined as the amount of enzyme required to cause the oxidation of 1 n mole of NADPH per min under the assay conditions described.

Kinetic Analysis of S. mansoni Peroxiredoxins—Detailed steady-state kinetic analysis was performed using the assay described above with the conditions described by Dalziel (22). Concentrations of EcTrx (4–100 μM), GSH (100–500 μM), and hydroperoxide (50–500 μM) were selected.

Overoxidation of S. mansoni Peroxiredoxins—Trx-dependent NADPH oxidation expressed as decrease in A410 was monitored at 25 °C for Prx1 and C-truncated Prx2 with H2O2 concentrations of 0.1–30 mM and for Prx2 and tailed-Prx1 with H2O2 concentrations of 0.1–2.5 mM. The rate of NADPH consumption at each time interval was expressed as micrograms of NADPH consumed per minute. The rate of the initial enzyme rate and the rate of the initial enzyme rate, V0, was calculated by plotting the slopes of rates versus H2O2 concentrations as described (23).

RESULTS

Peroxiredoxins of S. mansoni—Screening the existing S. mansoni EST data base identified two novel 2-cysteine Prx genes that were cloned and sequenced. Sequences of Prx1 (12), Prx2, and Prx3 were verified by sequencing genomic clones identified in the S1 baculovirus library (24) and submitted to GenBankTM with the accession numbers AF301002–AF301004 (data not shown). The three predicted schistosome Prx proteins are ~60% identical to each other (Fig. 1) and to Prx from numerous other organisms. Conservation of both peroxidatic
and resolving cysteines and neighboring amino acids places them in 2-Cys Prx family (11). Prx3 is predicted to be a mitochondrial protein with a probability of export to mitochondria of 0.85 and a cleavage site after Ala33 (25). All S. mansoni Prx proteins and mutants were expressed at high levels in E. coli (Fig. 2). Several proteins were expressed in inclusion bodies and were effectively solubilized in 5% (v/v) monothioglycerol (tailed-Prx1, Prx2, Prx2 C168S, and Prx3) or in 5% monothioglycerol plus 10% (v/v) glycerol (C-truncated-Prx2).

Activities of S. mansoni Peroxiredoxins with Different Reducing Equivalent System—The specific activities of the different Prxs were determined with both reducing equivalent systems (Fig. 3). Prx2 and Prx3 are highly active with Trx and GSH, the first two-Cys peroxiredoxins to have such activity. The activity with GSH appears to be conferred by the C-terminal portion of the protein as tailed-Prx1; Prx1, with the 22 C-terminal amino acids of Prx2, is also highly active with GSH. As shown previously (12), Prx1 has ~10% of the activity with GSH compared with Trx. The newly identified Prx2 and Prx3 were found to be more active with cumene hydroperoxide and t-butyl hydroperoxide than Prx1.

Steady-state Kinetic Analysis of Recombinant S. mansoni Prx—Peroxiredoxins characterized to date have non-saturable behavior typical of a double displacement (ping-pong) mechanism (11). The kinetic mechanism of parasite Prx1 was determined by single curve progression analysis (22) at several fixed concentrations of E. coli Trx. The H2O2 concentration was selected such that the assay rate was dependent on its concentration. The reciprocal initial velocities multiplied by the enzyme molar concentration were plotted against the reciprocal concentrations of H2O2. These plots yielded parallel lines (Fig. 4A), which implies non-saturable kinetics as described by Dalziel equation for bi-substrate reactions as shown in Equation 1,

$$[E]_{\text{act}} = [E]_{\text{act}} + [E]_{\text{act}}[A] + [E]_{\text{act}}[B] \quad (\text{Eq. 1})$$

where $[E]_{\text{act}}$, [A], and [B] are the molarities of enzyme, hydroperoxide, and reduced thioredoxin. In this case ternary complexes between the enzymes and both substrates ($[E]_{\text{act}}[A][B]$) are not formed during the catalysis. The ordinate intercepts represent apparent maximum velocities for infinite H2O2 concentrations at defined concentrations of Trx. In the secondary plot (Fig. 4B) these intercepts are plotted against the reciprocal Trx concentrations to yield the real maximum velocity. The real maximum velocity of Prx1 was determined to be infinite (Table I). φ0 was found to be zero, indicating that enzyme saturation by substrate cannot be achieved. Therefore, Prx1 catalysis looks like two independent reactions. First, the reduced Prx1 is oxidized by a hydrogen peroxide (Reaction 1),

$$\text{Prx1}_{\text{red}} + \text{HOOH} \rightarrow \text{Prx1}_{\text{red}} + \text{H}_2\text{O}$$

and

$$\text{Prx1}_{\text{red}} + \text{Enzyme} \rightarrow \text{Prx1}_{\text{oxd}} + \text{Enzyme}_{\text{oxd}} + \text{H}_2\text{O}$$

Second, the reduced enzyme is regenerated again by reduced thioredoxin (Reaction 2),

$$\text{Prx1}_{\text{red}} + \text{Trx}_{\text{red}} \rightarrow \text{Prx1}_{\text{red}} + \text{Trx}_{\text{red}} + \text{H}_2\text{O}$$

The Dalziel coefficients $\Phi_1$ and $\Phi_2$ are the reciprocal values of the apparent net forward rate constants $k_1'$ and $k_2'$, respectively (Table I).

As indicated earlier, Prx2 and Prx3 are active with both thiol-reducing systems (Fig. 3). Prx2 and Prx3 exhibit saturable, Michaelis-Menten-type kinetics, in which saturation conditions were achieved with different hydroperoxide substrates tested. Michaelis constants ($K_m$), catalytic constants ($k_{cat}$), and catalytic efficiencies ($k_{cat}/K_m$) were determined (Tables II and III). The $K_m$ for E. coli Trx was ~9 μM and ~200 μM for GSH for both Prx2 and Prx3. The $K_m$ for hydroperoxide substrates was dependent on the thiol-reducing agent present. Prx2 had a higher affinity for hydroperoxides in the presence of GSH ($K_m$ 2–5 times lower with GSH than with Trx), whereas Prx3 had a higher affinity for hydroperoxides in the presence of Trx ($K_m$ 1.3–6 times lower with Trx than with GSH). The catalytic efficiencies of Prx2 and Prx3 were similar to those for other peroxiredoxins (10^4–10^5 M^-1 s^-1) (15). Catalytic efficiencies were generally higher with GSH than with Trx for both Prx2 and Prx3 resulting primarily from a higher catalytic rate ($k_{cat}$) with GSH than with Trx. Affinity for the three hydroperoxide substrates were similar for Prx2 and Prx3, with a slight de-
crease in activity (H$_2$O$_2$ > cumene hydroperoxide > t-butylhydroperoxide) seen with Prx2. Further analysis indicated that Prx2 (results not shown) and Prx3 indeed displayed a single displacement-like mechanisms (Fig. 5). Significant inhibition of a Prx2 and Prx3 activity at higher concentrations of hydroperoxide substrate was seen (results not shown).

Kinetic Analysis of Recombinant Mutant S. mansoni Prx—To understand the unusual activity of both Prx2 and Prx3 with the GSH/GR system and their saturable kinetic behavior, we generated three mutant enzymes. A significant difference between Prx1 and Prx2/Prx3 is the presence of a C-terminal extension in Prx2 and Prx3 that is absent in Prx1. In modeling studies the C-terminal extension is thought to form an additional α-helix (23). Swapping of the 22 C-terminal amino acids of Prx2 to Prx1 (tailed-Prx1) changed the reaction kinetics of Prx1 to more closely resemble those of Prx2. Tailed-Prx1 exhibited substrate saturation typical of Michaelis-Menten type kinetics with similar kinetic constants and catalytic efficiencies to Prx2 (Tables II and III) and similar and high activities with both Trx and GSH thiol reducing systems (Tables II and III, and Fig. 3).

Deletion of the 21 C-terminal amino acids of Prx2 (C-truncated Prx2) resulted in an enzyme with non-saturable kinetics and activity restricted to the Trx-reducing system similar to Prx1. Dalziel constants for C-truncated Prx2 were found to be similar to Prx1 (Table I).

It has been determined that certain 1-Cys peroxiredoxins use GSH instead of Trx (26, 27). Because schistosome Prx2 and Prx3 function better with GSH than with Trx, we wanted to determine if these two enzymes functioned more like 1-Cys Prx than 2-Cys Prx. Therefore, we generated a mutant Prx2 by PCR-directed mutagenesis. The substitution of Cys$^{168}$ with Ser (C168S) yielded a completely inactive enzyme with either the GSH/GR system (results not shown). This indicates that Prx2 functions as a 2-Cys Prx.

Sensitivity of S. mansoni Prx to Overoxidation—During the Prx catalytic cycle, the peroxidatic cysteine (-SH) is oxidized to a sulfenic acid (-SOH), which then condenses with the resolving cysteine to form a disulfide, which is in turn reduced by thioredoxin, GSH, or another enzyme (8, 14, 15, 28). Under highly oxidative conditions, Prx can become inactivated by overoxidation of the peroxidatic cysteine to sulfonic acid (-SO$_2$H), which may allow peroxide signaling to proceed (23, 29). Two conserved regions in Prx sensitive to overoxidation were identified, a 3-residue insertion in the loop between α-helix 4 and β-sheet 5 associated with a conserved Gly-Gly-Leu-Gly sequence (GGLG) and an additional helix in a C-terminal extension with the conserved sequence Tyr-Phe (YF) (23). Comparison of the schistosome Prx sequences (Fig. 1) indicated that all three contain the insertion/GGLG motif. However, Prx1 lacks the C-terminal helix and the YF motif found in Prx2 and Prx3. We determined the resistance to overoxidation of Prx1 and Prx2.

**Fig. 3.** Relative activities of wild type and mutated peroxiredoxin under Trx/TrxR (A) and GSH/GR (B) systems with different hydroperoxide substrates. Bars indicate the specific activity (nanomoles of NADPH/min/mg of protein) of peroxiredoxins under Trx/TrxR and GSH/GR systems with different hydroperoxide substrates: H$_2$O$_2$ (black bars), cumene hydroperoxide (gray bars), and t-butylhydroperoxide (open bars). The specific activity of each enzyme was determined at 500 μM of each hydroperoxide substrate. n.d., no activity detected.

**Fig. 4.** Kinetic analysis of S. mansoni recombinant Prx1 as described by Dalziel (22). In the primary plot (A) the enzyme-normalized reciprocal initial velocities at four different concentrations of reduced thioredoxin: 4 μM (closed diamonds), 8 μM (closed square), 16 μM (closed triangle), and 50 μM (open square) are plotted against the reciprocal molarities of hydrogen peroxides. The ordinate intercepts of the primary plot (A), representing apparent maximum velocities, are then plotted against the reciprocal molarities of reduced thioredoxin reductase. Dalziel coefficients are obtained from slopes and intercepts as described under “Experimental Procedures.” The ordinate intercepts of the primary plot (A), representing apparent maximum velocities, are plotted against the reciprocal molarities of reduced thioredoxin. The plot (B) intercepts the ordinate at zero, indicating that V$_{max}$ is infinite.
Prx1 was found to be a robust enzyme, tolerating high concentrations of hydroperoxide, whereas Prx2 was sensitive to overoxidation (Fig. 6); the enzymes differed in sensitivity by a factor ~20. To further understand the mechanisms of overoxidation sensitivity, we assessed the sensitivity of the tailed Prx1 containing the C-terminal helix and YF motif to overoxidation. Tailed Prx1 was found to be as sensitive to overoxidation as Prx2 (Fig. 6). Removal of the C-terminal α-helix and the YF motif from Prx2 resulted in a protein with a resistance to overoxidation similar to Prx1 (Fig. 6).

**DISCUSSION**

Adult *S. mansoni* live in the hepatic mesenteries of their human host and avoid damage from reactive oxygen intermediates without catalase and with GPx that is more active toward phospholipid hydroperoxides than H$_2$O$_2$. The recent finding that a bacterial Prx is the primary scavenger of H$_2$O$_2$ indicates the importance of these enzymes in redox balance. We have recently shown that *S. mansoni* recombinant Prx1 is sensitive to overoxidation similar to Prx1 (Fig. 6).

**Data are the means of three independent measurements.**

| Enzyme (thiol system) | Substrate | $K_m$ | $k_{cat}$ | $k_{cat}/K_m$ | $k_{cat}/K_m$ |
|-----------------------|-----------|------|---------|-------------|-------------|
|                       | Hydroperoxide |      |         |             |             |
|                       | Cumene hydroperoxide |      |         |             |             |
|                       | t-Butylhydroperoxide |      |         |             |             |
| SmPrx1 (Trx/TR)       | H$_2$O$_2$ | 0    | 3.5     | 16          | ~          |
|                       | Cumene OOH | 0    | 1.3     | 20          | ~          |
|                       | t-hOOH    | 0    | 1.2     | 4.5         | ~          |
|                       | H$_2$O$_2$ | 0    | 5.7     | 0.4         | ~          |
| SmPrx1 (GSH/GR)       | Cumene OOH | 0    | 2.1     | 1.0         | ~          |
|                       | t-hOOH    | 0    | 2.9     | 0.1         | ~          |
|                       | C-truncated Sm Prx2 (Trx/TR) | H$_2$O$_2$ | 0    | 4.5     | 20          |
|                       | Cumene OOH | 0    | 2.8     | 27          | ~          |
|                       | t-hOOH    | 0    | 1.3     | 8.0         | ~          |

**Saturation kinetic constants for wild type and mutated *S. mansoni* peroxiredoxins and hydroperoxide dependence**

Kinetic parameters $K_m$ ($\mu$m) and $k_{cat}$ (s$^{-1}$) were determined using kinetic NADPH consumption assay at 340 nm. The reaction was conducted with GSH/GR or Trx/TrxR as reducing equivalent donors and with H$_2$O$_2$ (500 $\mu$m). A range from 10 $\mu$m to 1 m$m was used for hydroperoxide substrates.

| Enzyme                  | Substrate                   | Hydroperoxide | Cumene hydroperoxide | t-Butylhydroperoxide |
|-------------------------|-----------------------------|---------------|----------------------|----------------------|
|                         | $K_m$ | $k_{cat}$ | $k_{cat}/K_m$ | $K_m$ | $k_{cat}$ | $k_{cat}/K_m$ | $K_m$ | $k_{cat}$ | $k_{cat}/K_m$ |
| SmPrx2                  | GSH  | 99    | 19 $s^{-1}$   | 2 $\times 10^5$   | 80   | 8.2 $s^{-1}$ | 1 $\times 10^5$   | 135  | 9.2 $s^{-1}$ | 7 $\times 10^4$   |
|                         | Trx  | 215   | 9.8 $s^{-1}$ | 5 $\times 10^4$   | 428  | 4.9 $s^{-1}$ | 1 $\times 10^4$   | 580  | 4.5 $s^{-1}$ | 7 $\times 10^3$   |
| SmPrx3                  | GSH  | 316   | 41 $s^{-1}$  | 1 $\times 10^4$   | 296  | 9.6 $s^{-1}$ | 3 $\times 10^4$   | 320  | 8.6 $s^{-1}$ | 3 $\times 10^4$   |
|                         | Trx  | 166   | 4.0 $s^{-1}$ | 2 $\times 10^4$   | 231  | 3.0 $s^{-1}$ | 1 $\times 10^4$   | 52   | 2.2 $s^{-1}$ | 4 $\times 10^3$   |
| Tailed SmPrx1           | GSH  | 82    | 20 $s^{-1}$  | 1 $\times 10^4$   | 62   | 7.6 $s^{-1}$ | 1 $\times 10^4$   | 116  | 7.0 $s^{-1}$ | 6 $\times 10^3$   |
|                         | Trx  | 316   | 5.5 $s^{-1}$ | 1 $\times 10^4$   | 399  | 2.9 $s^{-1}$ | 7 $\times 10^3$   | 413  | 3.3 $s^{-1}$ | 7 $\times 10^3$   |

**Saturation kinetic constants for wild type and mutated *S. mansoni* peroxiredoxins, thiol dependence**

Kinetic parameters $K_m$ ($\mu$m) and $k_{cat}$ (s$^{-1}$) were determined using kinetic NADPH consumption assay at 340 nm. The reaction was conducted with GSH/GR or Trx/TrxR as reducing equivalent donors and with H$_2$O$_2$ (500 $\mu$m). A range from 10 $\mu$m to 1 m$m was used for thiol substrates.

| Enzyme                  | Substrate                   | Thioredoxin | Glutathione |
|-------------------------|-----------------------------|-------------|-------------|
|                         | $K_m$ | $k_{cat}$ | $k_{cat}/K_m$ | $K_m$ | $k_{cat}$ | $k_{cat}/K_m$ |
| SmPrx2                  | 8.7   | 4.3     | 5 $\times 10^5$ | 216  | 10.3    | 4 $\times 10^4$ |
| SmPrx3                  | 9.5   | 3.3     | 3 $\times 10^5$ | 195  | 20.2    | 1 $\times 10^5$ |
| Tailed SmPrx1           | 7.3   | 4.2     | 6 $\times 10^5$ | 234  | 9.1     | 4 $\times 10^4$ |
tain GSH in the reduced state, in a manner similar to what has been described in dipteran insects (34, 35). Trx and GSH would then supply reducing equivalents to Prx and GPx for the reduction of hydroperoxides. Further blending of the two redox pathways is seen with Prx2 and Prx3 using both GSH and Trx as reducing agents.

*S. mansoni* Prx1 has a non-saturatable kinetic behavior as described by Dalziel (22) as is the case with the classic GPxs (36) and peroxiredoxins studied to date (11). The real maximum velocity of Prx1 was determined to be infinite suggesting that enzyme saturation by hydroperoxide substrates cannot be achieved. On the other hand, typical Michaelis-Menten saturable kinetics was obtained for both *S. mansoni* Prx2 and Prx3 under both GSH and Trx systems. Catalytic rates for schistosome Prxs were similar to those seen for Prx from other organisms (34, 35) and significantly lower than those of GPx (36) and catalases (36) (11).

To better understand the unusual GSH dependence of schistosome Prx, three different mutant enzymes were generated. The 21-amino acid C-terminal tail of Prx2 was deleted resulting in complete abrogation of its GSH dependence while retaining its activity with Trx. Switching the 22-amino acid C-terminal helix from Prx2 to Prx1 (tailed Prx1) produced a chimeric bifunctional enzyme, which was kinetically and mechanistically similar to Prx2. Because some 1-Cys Prx have been shown to use GSH (26, 27) we wondered if Prx2 functioned by mechanisms similar to 1-Cys Prx. The amino acid residues surrounding the Cys48 and Cys168 of *S. mansoni* Prx2 are conserved compared with Prx from other organisms. Therefore, we expected Cys48 to be the peroxidatic Cys and Cys168 to be the resolving Cys as in other Prx enzymes. Cys48 is essential for the activity of both 1-Cys and 2-Cys Prx, whereas Cys168 is essential only for 2-Cys Prx and absent in 1-Cys Prx. If a 2-Cys-type reaction cycle occurred both Cys48 and Cys168 would be essential for the reaction cycle of SmPrx2. If a 1-Cys-type reaction cycle occurred, Cys168 would be irrelevant in the reaction cycle of SmPrx2. To address this a mutant enzyme obtained by replacing the second conserved Cys168 of Prx2 with Ser (C168S) was generated. Prx2 C168S was inactive with both GSH and Trx systems indicating that Prx2 is a 2-Cys Prx. Further analysis by point mutagenesis of residues in the C-terminal domain of Prx2 could identify the mechanism by which Prx2 utilizes GSH.

Signal transduction and gene regulation through H2O2 signaling has gained considerable attention recently (37–40). Prxs have been shown to regulate H2O2 signaling as well as acting as antioxidants. Prxs function to maintain low resting levels of hydrogen peroxide. During signaling when H2O2 levels rise, Prxs are inactivated allowing signal transduction to occur effectively acting as a molecular floodgate (23, 41). Because bacteria do not appear to use oxidative signaling mechanisms, bacterial Prx are resistant to oxidative inactivation. Wood and coworkers (23) postulated that the loop containing the GGGLG motif and the C-terminal helix containing the YF motif pack next to each other and bury the active site helix containing the cysteine. The C-terminal helix with its conserved, buried YF motif results in tighter structure serving as a keystone that strongly stabilizes this whole region locking the oxidized enzyme and preventing easy reduction of the peroxidatic cysteine as sulfenic (-SOH), which can then be oxidized by the hydroperoxide to the inactive sulfinic acid (-SO2H) form (23). Analysis of the schistosome Prx sequences indicated that the insertion and GGGLG motif is present in all three schistosome Prxs, whereas the C-terminal helix and its YF motif are present in Prx2 and Prx3 but not in Prx1. It has also been proposed that the regulation of Prx activity involves specific proteolysis of the C termini of Prxs preventing peroxide-mediated inactivation (42).

We have determined that schistosome Prx1 is resistant to overoxidation, whereas Prx2 is sensitive to overoxidation (Fig. 6). Furthermore, transfer of the C-terminal 22 amino acids, including the YF motif of Prx2 to Prx1, converts it into an overoxidation-sensitive enzyme. Likewise, C-truncated Prx2, which lacks the C-terminal helix and YF motif, is a robust enzyme, with resistance to oxidative inactivation similar to Prx1. Our results support the hypothesis of Wood and coworkers (23).

*S. mansoni* appears to constitutively express a resistant Prx and to express elevated levels of this protein in its eggs (13). During the life cycle of the parasite, eggs released from female worms are enveloped in an immune-generated granuloma. Neutrophils and eosinophils present in the granuloma generate high levels of reactive oxygen intermediates around the egg (43, 44). Although these immune cells can eventually destroy liver-trapped eggs and are the main cause of host pathology, formation of the granuloma is, paradoxically, essential for the passage of the egg across the intestinal wall and for survival of the host (44–47). It is of note that schistosome Prx1 was found in egg secretions as well as its required reducing agent Trx (13, 48). The activity of antioxidant proteins, such as Prx and Trx, in the egg may be essential to prevent oxidative destruction...
FIG. 6. Resistance to inactivation by H$_2$O$_2$ analysis (overoxidation). In A: time course of Trx-dependent NADPH oxidation expressed as decrease in $A_{340}$ was monitored at 25 °C for Prx1 (graph a) and C-truncated Prx2 (graph g) with H$_2$O$_2$ concentrations of 0.1 mM (closed diamonds), 1 mM (closed square), 5 mM (closed triangle), 10 mM (open square), and 30 mM (open diamond). Graphs c and e, time course Trx-dependent NADPH oxidation expressed as decrease in $A_{340}$ was monitored at 25 °C for Prx2 and tailed Prx1, respectively, with H$_2$O$_2$ concentrations of 0.1 mM (closed diamonds), 0.25 mM (closed square), 0.5 mM (closed triangle), 1 mM (open square), and 2.5 mM (open diamond). Graphs b, d, f, and h, the rate of NADPH at each time point expressed as the percentage of the initial enzyme rate ($t = 0$) for (graphs a, c, e, and g) curves, respectively. In B: peroxide dependence of inactivation of Prx1, Prx2, tailed Prx1, and C-truncated Prx2. The rate of inactivation was calculated by plotting the slopes of curves in A, graphs b, d, f, and h versus H$_2$O$_2$ concentrations; Prx1 (closed square), Prx2 (closed triangle), Tailed Prx1 (open square, broken line), and C-truncated Prx2 (open triangle, broken line).
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