The apicomplexan parasite *Toxoplasma gondii* is highly susceptible to oxidative stress caused by tert-butyl-hydroperoxide, juglone, and phenazine methylsulfate with IC$_{50}$ in the nanomolar range. Using dichlorofluorescein diacetate, a detector of endogenous oxidative stress, it was shown that juglone and phenazine methylsulfate are potentially toxic to the parasites without affecting the host cells. These results demonstrate that *T. gondii* is vulnerable to oxidative challenge that results from disruption of its redox balance and so this could be an effective approach to therapeutic intervention. This study has characterized redox active and antioxidant peroxidases belonging to the class of 1-Cys and 2-Cys peroxiredoxins that play crucial roles in maintaining redox balance. The tachyzoite stages of *T. gondii* express thioredoxin (TgTrx), 1-Cys peroxiredoxin (TgTrx-Px2), and a 2-Cys peroxiredoxin (TgTrx-Px1) and immunofluorescent studies revealed that all three proteins are located in the cytosol of the parasite confirming previous studies on TgTrx-Px1 (Kwok, L.Y., Schlueter, D., Clayton, C., and Soldati, D. (2004) *Mol. Microbiol.* 51, 47–61). TgTrx-Px1 showed $K_m$ values for H$_2$O$_2$ and tert-butyl hydroperoxide in the nanomolar range, emphasizing the great affinity of the protein for these substrates. Moreover, the catalytic efficiency of TgTrx-Px1 for these substrates at $10^{-6}-10^{-7}$ M depending on the concentration of the non-inhibitory substrate thioredoxin. TgTrx-Px2 protected glutamine synthetase from inactivation by Fe$^{3+}$/DTT, showing that it is an active peroxiredoxin.

Peroxiredoxins (Trx-Px) are a family of antioxidant enzymes that protect cells from oxidative damage by hydroperoxides (1, 2). The enzymes are peroxidases that exert their reductive activity via active site cysteine residues. This group of proteins can structurally be distinguished into three classes: the typical 2-Cys peroxiredoxins, the atypical 2-Cys peroxiredoxins, and the 1-Cys peroxiredoxins (2). Atypical 2-Cys peroxiredoxins have only been identified recently and they differ from typical 2-Cys peroxiredoxins by forming an intramolecular disulfide bridge during their catalytic cycle rather than an intermolecular disulfide bridge like typical peroxiredoxins (2, 3). The abundance of typical 2-Cys peroxiredoxins can be extraordinarily high, possibly compensating for their rather low catalytic efficiency when compared with other peroxidases such as catalase and glutathione-dependent peroxidases (1, 2). In addition to their protective antioxidant role, it has been suggested that 2-Cys peroxiredoxins are involved in redox signaling and other regulatory processes (2, 4–6). Thus this class of enzymes has a wide variety of functions that are vital for metabolism and cellular integrity. The role of the 1-Cys peroxiredoxins is less clear. Their catalytic mechanism has hardly been studied to date, and the nature of their endogenous reducing partner is controversial (3, 7–9).

*Toxoplasma gondii* causes toxoplasmosis in warmblooded animals including humans. An infection with *T. gondii* can be lethal in immunocompromised individuals and presents a particular threat to patients with AIDS/HIV. In Africa, where toxoplasmosis is the cause of death in 10–30% of AIDS patients (10). In addition, an infection with *T. gondii* in pregnant women leads to the congenital infection of the fetus (11) affecting the central nervous system and leading to a variety of severe disorders. Recently the antioxidant systems of *T. gondii* have started to attract attention as these might be essential for the adaptation and survival of the parasites in macrophages and other immune effector cells, which probably generate reactive oxygen species to combat the parasites (12, 13). Thus *Toxoplasma* needs to have effective antioxidant systems to maintain the intracellular redox homeostasis even when the parasite is under oxidant challenge. In previous studies it has been shown that *T. gondii* possesses a whole array of antioxidant proteins including three superoxide dismutases, catalase, and a variety of putative glutathione- and thioredoxin-dependent peroxidases of the peroxiredoxin family (13–16). The presence of catalase is unusual as other apicomplexan parasites such as *Plasmodium* (17), and the members of the kinetoplastida lack this protein and other antioxidants appear to compensate for its absence (18, 19). As the disruption of the catalase gene in *T. gondii* was found to be non-lethal, the enzyme presumably does not have a vital role in the parasite (13). It is feasible that the parasite peroxiredoxins compensate for the
loss of catalase and indeed the non-essentiality of catalase suggests that this group of proteins represents the major anti-
oxidant defense system in Toxoplasma. Indeed, it has been shown that the thioredoxin-dependent reduction of hydroper-
oxides is of vital importance in the related apicomplexan parasi-
tae Plasmodium falciparum as thioredoxin reductase is es-
sential for survival of this parasite (20). In this study we have charac-
terized the peroxiredoxin proteins of T. gondii, and the results suggest that the systems involving these proteins play key roles in protecting the parasite against oxidative damage.

**EXPERIMENTAL PROCEDURES**

**Materials**—FITC-labeled secondary anti-rat and anti-rabbit antibod-
ies were obtained from Molecular Probes, Leiden, The Netherlands. Poly
clonal antibodies were raised against T. gondii thioredoxin (TgTrx) in a rat, T. gondii 1-Cys peroxiredoxin (TgTrx-Px2) and 2-Cys peroxi-
redoxin (TgTrx-Px1) in rabbits by Eurogentec (Belgium). Genomic and cDNA libraries of T. gondii were obtained from the National Institutes of Health. [3H]Uracil (48 Ci/mmol) was purchased from Amersham Biosciences. F. paralpum thioredoxin reductase was expressed and purified according to Gilberger et al. (21). F. paralpum thioredoxin was expressed and purified according to Kranjsci et al. (22). Minimal essential medium (MEM), Dulbecco’s modified Eagle’s medium, RPMI 1640, penicillin/streptomycin, and fetal calf serum were from Invitrogen. Glutamine synthetase from Escherichia coli was purchased from Sigma. Human foreskin fibroblasts (HFFs) were from LGC Promochem (Teddington, UK).

**T. gondii Culture and Determination of IC₅₀ of Oxidative Stressors**—T. gondii tachyzoites (RH strain) were maintained on monolayers of HFFs in MEM containing 1% fetal calf serum at 37 °C in 5% CO₂ according to Ref. 23. In order to determine the susceptibility of T. gondii to the oxidative stressors tert-butyli-hydroperoxide (t-ButOOH), 5-hydroxy-1,4-naphthoquinone (juglone; Fluka) and the superoxide-gener-
ating compound phenazine methosulfate (Fluka) a monolayer of HFFs was grown in 24-well plates, infected with 1 × 10⁵ parasites per well, and treated for 3 h with the concentrations of the stressors indicated (see Table 1). After 24 h, the media were supplemented with [3H]uracil (2.5 μCi/well) and incubated for an additional 4 h. Then the cells were treated with 0.6 M trichloric acid for 1 h and subsequently washed extensively with excess water overnight. Then the cells were solubilized for 1 h with 500 μl of 0.1 M NaOH before 250 μl of each well are transfered into scintillation vials containing 3 ml of Picofluor scintil-
lation fluid (Packard Biosciences) and the incorporation of [3H]uracil into the ribonucleic acids of the parasites determined by scintillation counting (Beckman LS6500). In a control experiment, non-infected HFFs were treated with increasing concentrations of the three stressors under the same conditions as described for the parasitized cells, and their viability was determined using a Live/Dead assay (Molecular Probes) and subsequent analysis by fluorescent microscopy with an Axioscope 200 M microscope (Zeiss).

**Isolation of Nucleic Acids and Proteins**—In order to isolate genomic DNA and total RNA from T. gondii tachyzoites, they were grown to high density and isolated from lysed HFFs using 0.6 μM Nuclease Track-
Etch membranes (Whatman). Parasites were pelleted and either resus-
pended in TE buffer (10 mM Tris, pH 7.5, 1 mM EDTA) (for DNA isolation) or TRIzol (Invitrogen) for RNA isolation. In order to obtain parasite proteins, the isolated parasites were resuspended in PBS con-
taining EDTA-free protease mixture (Roche Applied Science) and sub-
sequently lysed by several cycles of freeze-thawing followed by sonication.

**Cloning of TgTrx-Px1 (2-Cys Peroxiredoxin), TgTrx-Px2 (1-Cys Peroxiredoxin), and TgTrx—Searching the t. gondii genome data base (www.ToxoDB.org) with the translated amino acid sequences of P. falciparum Trx, P. falciparum Px1 (2-Cys peroxiredoxin) and P. falciparum Px2 (1-Cys peroxiredoxin) revealed the presence of homologues of the three Plasmodium proteins. Oligonucleotides TgTrx-ISense and TgTrx-IAn-
tisense (5’-GCCCAATATGCGGCAGTCATCGACGAC-3’ and 5’-GCC-
GACATACCAGTTTGTGTTGCT-3’) were used to amplify the coding regions of the three genes with Pfu polymerase and a T. gondii cDNA library as template. The condi-
tions for the polymerase chain reaction (PCR) were as follows: 1 cycle at 95 °C for 1 min and 30 cycles of 1 min at 95 °C, 1 min 50 to 60 °C, and 1 min at 72 °C followed by an extension cycle at 72 °C for 7 min. The PCR products were cloned into TOPO blunt (Invitrogen), and their sequences were determined by The Sequencing Service (School of Life Sciences, University of Dundee, Scotland, www.dnaseq.co.uk) using Applied Biosystems Big-Dye Ver 3.1 chemistry on an Applied Biosys-
tems model 3730 automated capillary DNA sequencer. In order to re-
combinantly express the three T. gondii proteins the inserts of the recom-
binant clones were isolated by digestion with NdeI, BamHI, and XhoI as required and subcloned into pUC40 previously digested with the respective restriction enzymes. The sequence of the expression vector was confirmed and used for transformation into E. coli BLR (DE3)-pLys (Novagen).

**Recombinant Expression of TgTrx-Px1, TgTrx-Px2, and TgTrx—** Bacteria harboring the expression plasmids were used to inoculate overnight cultures in Luria-Bertani medium containing 50 μg ml⁻¹ ampicillin. The cultures were diluted 1:50 and bacteria grown at 37 °C until their OD₆₀₀ reached 0.5 before recombinant protein was induced using 1 mM isopropyl-β-thio-galactoside. The bacteria were cooled to 27 °C after induction and grown for an additional 18 h before they were harvested at 3500 × g (Beckman J6). Bacterial pellets were resuspended in 50 mM sodium phosphate buffer, pH 8.0 containing 1000 μM NaCl and 10 mM imidazole (lysing buffer) and lysed by adding 50 μg ml⁻¹ lysozyme, freeze-thawing, and by using a French Press (1000 pounds inch⁻²; American Instrument Company). The bacterial extract was cleared by centrifugation for 1.5 h at 50,000 × g (Beckman, Avanti, JA-25). The resulting supernatants were applied to Ni²⁺-agarose, and the subsequent purification of the recombinant proteins was performed in a batch procedure according to the manufacturer’s instructions (Qiagen). Protein concentrations were determined using the Bradford method (24) with bovine serum albumin as standard.

**Enzyme Assays for Thioredoxin—** To test whether T. gondii Trx is a competent substrate of P. falciparum thioredoxin reductase (PfTrxR), the TrxR activity was determined spectrophotometrically using three different assay systems (1). In the insulin assay, the reduction of thioredoxin was coupled to the reduction of insulin as previously described (25, 26). The second assay was performed using a fast kinetics instrument (SFA-20, Hi-Tech Scientific) connected to the spectrophoto-
tometer (Shimadzu, 2401 PC) in order to determine the initial rates of Trx reduction (27). The assay mixture contained 200 μM NADPH, 100 μM HEPES pH 7.6, 1 μM EDTA, 1 μg of PfTrxR, and increasing concentrations of TgTrx. Time points were taken every 10 ms, and the initial rates were used to determine the steady-state kinetic parameters for the reduction of both parasite Trxs (3). The third assay system was performed in order to test whether TgTrx also reduces glutathione disulfide (GSSG) as has been previously reported for the proteins from P. falciparum and Drosophila melanogaster (28, 29). The assay conditions were as follows: 5–10 μg of PfTrxR, 1 to 10 μM TgTrx or PfTrx, and 0.025–2 mM GSSG in 100 μM HEPES pH 7.6, 1 mM EDTA, and 200 μM NADPH. The change in absorbance was monitored spectrophotometrically at 340 nm, and the results were used to calculate the second order rate constant k₂ according to Equation 1.

\[ v = k_2 [Trx(SH)₂] [GSSG] \]  

(Eq. 1)

Further whether TgTrx and PfTrx are reduced by dihydroilopamide dehydrogenase (LidDH) and dihydroilopamide was tested. The assays systems contained 200 μM NADH, 5 units of dihydroilopamide dehy-
drogenase (a. 25 μM lidocaine), 1 μM EDTA, 10 mM HEPES pH 7.6, 10 to 50 μM of TgTrx and PfTrx, and 0.2 mg ml⁻¹ insulin, and the change in absorbance was monitored at 340 nm on a Shimadzu 2401 PC spectrophotometer. The second order rates for the reduction of both parasite Trxs by dihydroilopamide were calculated according to Equation 2.

\[ v = k_2 [Trx(S)_2] [lipoiamide disulfide] \]  

(Eq. 2)

**Peroxiredoxin Assays—** The T. gondii 2-Cys peroxiredoxin kinetic parameters were determined according to Akerman and Muller (27) using a spectrophotometric assay employing stopped-flow fast kinetics.
with 15 \(\mu\)g of PfTrxR, 1–25 \(\mu\)M TgTrx, 0.2 \(\mu\)M TgTrx-Px1, 100 mM Hepes pH 7.6, 1 mM EDTA, 200 \(\mu\)M NADPH, and 0.5–15 \(\mu\)M hydroperoxide substrate. Steady-state kinetic parameters for the interaction TgTrx-Px1 with hydroperoxide and tert-butyl hydroperoxide (0.5–50 \(\mu\)M) were determined, and the resulting data were fitted using Grafit 5.0.

**Substrates for TgTrx-Px2**—To establish the catalytic activity of TgTrx-Px2 the glutamate synthetase (GS) protection assay was utilized as described by Kim et al. (30). Because the endogenous reducing partner of *T. gondii* 1-Cys peroxiredoxins is elusive, a number of intracellular thiols, glutathione and dihydroliopamide and diithiol-containing peptides, thioredoxin and glutaredoxin from *P. falciparum* (31), were tested for their ability to act as a reductant for TgTrx-Px2. The assay system consisted of 1 mM GSH, 200 \(\mu\)M NADPH, 100 mM HEPES pH 7.6, 1 mM EDTA, 8–10 \(\mu\)g of *P. falciparum* glutathione reductase prepared according to Ref. 31, 0.5–5 \(\mu\)M TgTrx-Px2 and 100 \(\mu\)M H2O2. The glutaredoxin assay system essentially contained the same components as described above with the exception that 1–10 \(\mu\)M *P. falciparum* glutaredoxin, prepared according to Ref. 32, was included into the assay before the reaction was initiated by addition of H2O2. In the third assay system we established whether lipolic acid acts as a reductant for both parasite 1-Cys peroxiredoxins using the following assay conditions, 200 \(\mu\)M NADH, 5–50 \(\mu\)l lipopamine, 5 units of dihydroliopamide dehydrogenase (Sigma), 0.5–20 \(\mu\)M peroxiredoxin, and 100 \(\mu\)M H2O2. To test the ability of thioredoxin to act as a reducing thiol, a similar assay system as shown above with TgTrx-Px1 was performed where TgTrx-Px1 was replaced with TgTrx-Px2.

**Western Blotting**—Polyclonal antibodies were raised against TgTrx in rats and against TgTrx-Px1 and TgTrx-Px2 in rabbits (Eurogentec). The antibodies were purified according to standard protocols (33) and used to detect the three proteins in Western blots of *T. gondii* tachyzoites. The parasite protein extract was prepared on a 4–12 SDS-PAGE (Invitrogen) and subsequently blotted onto nitrocellulose. The blots were probed with the primary anti-TgTrx rabbit antibody at 1:1000 dilution, the anti-TgTrx-Px1 at 1:100 dilution, the anti-TgTrx-Px2 at 1:250 dilution, and with the anti-theresin IgG (DaKo; Scottish Antibody Production Unit) were applied at 1:5000, and the blots were developed using the ECL system (Amersham Biosciences).

**Immunofluorescence**—HFFs grown on chamber slides were infected with 0.5 \(\times\) 10^4 *T. gondii*, and after 24 h the cells were fixed with 4% paraformaldehyde for 5 min at 37°C and then permeabilized with 0.1% Triton X-100 for 5 min at room temperature. The wells were washed twice with PBS between each stage and blocked with 3% bovine serum albumin in PBS containing 0.05% Tween-20 and incubated overnight at 4°C in a humidity chamber. Subsequently the wells were washed three times with PBS, 0.05% Tween-20 prior to incubation with primary rat anti-TgTrx or purified rabbit anti-TgTrx-Px1 and purified rabbit anti-TgTrx-Px2 antibodies, respectively, at dilutions between 1:200 and 1:2000 for at least 1 h at room temperature. After several washes with PBS, the cells were incubated with a FITC-labeled anti-rat or anti-rabbit IgG (Molecular Probes) at a dilution of 1:500, incubated for 1 h, and after several washes slides were mounted using the slowfade light antifade kit containing 1.5 \(\mu\)g ml \(^{-1}\) DAPI (Molecular Probes) to stain the nucleic acids.

**RESULTS**

**Susceptibility to Oxidative Stress**—*T. gondii* tachyzoites were exposed to increasing concentrations of the exogenous oxidant tert-butyl hydroperoxide and inducers of oxidative stress (juglone, phenazine methsulafate) to determine their IC\textsubscript{50} values (concentrations that caused 50% inhibition of viability). The latter two compounds cause endogenous oxidative stress either by generating reactive oxygen species by redox cycling (35). Furthermore, the endogenous generation of superoxide anions in the presence of NADH or NADPH (34, 35). *T. gondii* were found to be highly sensitive to all three compounds with IC\textsubscript{50} values in the range between 100 and 500 \(\mu\)M (Fig. 1). They are most susceptible to tert-butyl hydroperoxide (IC\textsubscript{50} = 100 ± 8 \(\mu\)M) followed by juglone (IC\textsubscript{50} = 148 ± 16 \(\mu\)M) and phenazine methylsulfate (IC\textsubscript{50} = 406 ± 134 \(\mu\)M). At these concentrations, the viability of the host cells (HFFs) was not affected, as shown by incubating them by themselves under the same conditions as the parasite-infected HFFs and monitoring their viability with a Live/Dead assay system (data not shown). Indeed, the HFFs only start to show some response to the three stressors at concentrations 1 to 2 orders of magnitude higher than those affecting parasite growth.

Further investigation revealed that parasite death was very likely caused by increased oxidative stress. Incubation of *T. gondii*-infected HFFs with the stressors and subsequent monitoring of the occurrence of oxidative stress using DCF, which enters the cell and is converted into a fluorescent compound after reaction with H2O2 (36), revealed that juglone and phenazine methsulafate cause strong fluorescence in the parasites, whereas no fluorescence was detected in the control cells (Fig. 2). Interestingly, the host cells did not show any increased oxidative stress, suggesting that the stressors specifically affected the parasite.

**Sequence Analyses of TgTrx-Px1, TgTrx-Px-2, and TgTrx**—The genes encoding TgTrx-Px1 (2-Cys peroxiredoxin), TgTrx-Px2 (1-Cys peroxiredoxin), and TgTrx were amplified by PCR using a cDNA library as a template. The TgTrx-Px1 cDNA encodes a polyepptide of 196 amino acids and a theoretical molecular size of 21.7 \(\mu\)M with 52.0% identity to *P. falciparum* 2-Cys peroxiredoxin PfTrx-Px1 and 55.6% identity to Tp-Px from *Magnetococcus* (ZP_00043412), 53.6% identity to *T. gondii* TgTrx-Px2 and 100% identity to PfTrx-Px2 (P32119). TgTrx-Px1 contains two VCP motifs at positions 50–52 and 170–172, which is typical for the class of 2-Cys peroxiredoxins (6). In addition, the TgTrx-Px1 amino acid sequence possesses a GGGG sequence motif at positions 94–97 and a C-terminal extension containing a tyrosine residue at position 192. Both sequence motifs have been suggested to be involved in the susceptibility of eukaryotic 2-Cys peroxiredoxins to over-oxidation by hydroperoxides (6).

The TgTrx-Px2-deduced amino acid sequence encompasses 224 amino acids with a theoretical molecular size of 24.5 \(\mu\)M. TgTrx-Px2 possesses one VCPmotif at position 44–46 diagnostic for this redox-active protein (37). The gene encodes a 12.1 \(\mu\)M protein with a sequence identity of 47.6% to *P. falciparum* TgTrx (AJ277839.1) and 43.8% identity to Saccharomyces cerevisiae Tp (NP_013144).

**Recombinant Expression of TgTrx-Px1, TgTrx-Px-2, and TgTrx**—The three *T. gondii* proteins were recombinantly expressed in *E. coli* BLR (DE3), purified using Ni\textsuperscript{2+}-agarose, and their purity assessed by SDS-PAGE. All three proteins were
expressed at yields between 50 and 100 mg liter\(^{-1}\) bacterial culture. The subunit molecular sizes of the (His\(_{10}\))\(\text{-}\)tagged TgTrx-Px1, TgTrx-Px2, and TgTrx were determined to be 24.6 kDa, 26.5 kDa, and 15.7 kDa, respectively. Gel filtration on a previously equilibrated Sephadex S 200 column revealed that TgTrx-Px1 elutes as a high molecular mass species of \(\sim\)250 kDa, suggesting that the protein forms dimers as has been previously reported for 2-Cys peroxiredoxins from other organisms including \(P.\) falciparum (27). TgTrx-Px2 eluted in three peaks from the gel sizing column with a major peak corresponding to the size of the monomer of 25 kDa, and two minor peaks that correspond to molecular sizes of 52 kDa and 150 kDa, possibly representing homodimers and homohexamers of the protein. TgTrx eluted in a single peak corresponding to a monomer of 15 kDa. Antibodies were raised against the recombinant proteins and subsequently purified using standard protocols (33).

\(T.\) gondii Thioredoxin Is a Substrate for \(P.\) falciparum Thioredoxin Reductase—It was established that TgTrx is an effective substrate for PfTrxR with steady-state kinetic parameters similar to PfTrx (Table I). These results show that the two apicomplexan thioredoxins share key structural features that are distinct from the \(E.\) coli Trx, which is only a poor substrate for PfTrxR (38). Moreover, the finding that TgTrx was reduced well by PfTrxR facilitated the use of the \(Plasmodium\) reductase for this study.

In \(P.\) falciparum and insects, thioredoxin acts as redox shuttle between the thioredoxin and glutathione systems by reducing GSSG (28, 29). To assess if this could also occur in \(T.\) gondii, the second order rate constant \(k_{\text{red}}\) for the reduction of GSSG by TgTrx was determined (Table I). TgTrx efficiently reduced GSSG with a second order rate of \(2.5 \times 10^5\) \(\text{m}^{-1} \text{s}^{-1}\), which is 3 orders of magnitude lower than the second order rate for the reduction of insulin (25, 26) and in the same range as previously reported by Kanzok et al. (28) for the \(Plasmodium\) Trx (Table I). Interestingly, TgTrx and PfTrx not only connect the glutathione and thioredoxin redox cycles but also form a link to the dihydrolipoamide/lipoamide redox pair, as dihydrolipoamide efficiently reduces both parasite thioredoxins with second order rates of \(1.0 \times 10^4\) \(\text{m}^{-1} \text{s}^{-1}\) and \(1.2 \times 10^4\) \(\text{m}^{-1} \text{s}^{-1}\) for \(T.\) gondii and \(P.\) falciparum, respectively (Table I).

### Table I

| Features of apicomplexan thioredoxins | \(T.\) gondii | \(P.\) falciparum |
|--------------------------------------|---------------|------------------|
| \(K_m\) of PfTrxR                    | 2.7 \(\pm\) 0.5 \(\mu\)M | 6.5 \(\pm\) 1.4 \(\mu\)M |
| \(k_{\text{cat}}\) of PfTrxR         | 12.0 \(\text{s}^{-1}\) | 30.4 \(\text{s}^{-1}\) |
| \(k_{\text{red}}/K_m\) of PfTrxR     | \(4.4 \times 10^6\) \(\text{M}^{-1} \text{s}^{-1}\) | \(4.7 \times 10^6\) \(\text{M}^{-1} \text{s}^{-1}\) |
| \(k_{\text{red}}\) by dihydrolipoamide reduction of GSSG Subunit Mr |
| M                                     | 2.5 \(\times 10^5\) \(\text{M}^{-1} \text{s}^{-1}\) | 2.5 \(\times 10^5\) \(\text{M}^{-1} \text{s}^{-1}\) |
| Oligomeric state                      | 12,100         | 13,000           |

*a Results taken from Krajniki et al. (22) and Kanzok et al. (28). All other results are from this study.*
potential redox systems that might interact with TgTrx-Px2, none of glutathione, Trx, glutaredoxin, or lipoic acid reduced the recombinant TgTrx-Px2 protein. Thus, the endogenous, natural reductant for TgTrx-Px2 remains to be discovered.

Western Blotting and Immunofluorescence—The expression of the two peroxiredoxins and thioredoxin in the tachyzoite stages of *T. gondii* was analyzed by Western blotting. The results showed that they are all expressed in these parasite stages (Fig. 5). The antibodies were also used to determine the subcellular localization of TgTrx-Px1, TgTrx-Px2, and TgTrx by immunofluorescence. All three proteins are clearly located in the cytosol of the parasite (Fig. 6).

### DISCUSSION

It has been suggested that apicomplexan parasites such as *T. gondii* need efficient antioxidant systems in order to maintain the crucial balance between antioxidants and pro-oxidants to ensure the survival in their host cells (13). A number of antioxidant enzymes have been identified in *T. gondii* including superoxide dismutases and peroxiredoxins (13, 16, 43, 44). *T. gondii* possesses three peroxiredoxins, one 1-Cys peroxiredoxin and two 2-Cys peroxiredoxins (13). It seemed likely that these peroxiredoxins are important to *Toxoplasma*, especially as the parasite is able to survive the knockout of catalase (13).

### TABLE II

Features of *T. gondii* 2-Cys peroxiredoxins

|                     | *T. gondii* | *P. falciparum* |
|---------------------|-------------|-----------------|
| $K_m$ Trx           | 4.20 ± 0.90 μM | 13.4 ± 0.85 μM |
| $K_m$ H$_2$O$_2$    | 0.38 ± 0.03 μM | 0.25 ± 0.07 μM |
| $K_m$ t-BuOOH (5 μM Trx) | 0.040 ± 0.030 μM | ND$^b$ |
| $K_m$ t-BuOOH (2 μM Trx) | 0.040 ± 0.044 μM | ND |
| $k_{cat}$ H$_2$O$_2$ | 2.79 s$^{-1}$ | 1.67 s$^{-1}$ |
| $k_{cat}$ t-BuOOH (5 μM Trx) | 1.99 s$^{-1}$ | ND |
| $k_{cat}$ t-BuOOH (2 μM Trx) | 2.07 s$^{-1}$ | ND |
| $K_m$ Trx           | 2.2 × 10$^3$ M$^{-1}$ s$^{-1}$ | 6.7 × 10$^3$ M$^{-1}$ s$^{-1}$ |
| $K_m$ t-BuOOH (5 μM Trx) | 7.0 × 10$^3$ M$^{-1}$ s$^{-1}$ | ND |
| $K_m$ t-BuOOH (2 μM Trx) | 5.0 × 10$^3$ M$^{-1}$ s$^{-1}$ | ND |
| Catalytic mechanism | Ping-pong | Ping-pong |
| Subunit Mr           | 21,700 | 21,800 |
| Oligomeric state     | Dimer/Decamer | Dimer/Decamer |

$^a$ Results taken from Akerman and Mülle (27).

$^b$ ND, not determined.

**Fig. 3.** High substrate inhibition of TgTrx-Px1 by t-BuOOH. *A*, Michaelis-Menten plot of kinetic studies of TgTrx-Px1 (0.2 μM) with varying concentrations of t-BuOOH (0.5–50 μM) at constant TgTrx concentration of 2 and 5 μM that were carried out as described under “Experimental Procedures.” Every data point represents the mean of 3–5 independent measurements ± S.D. *B*, Lineweaver-Burk plot, the results were fitted to the high substrate inhibition equation (Equation 4) and $K_m^\text{app}$, $k_{cat}$, and $K_i$ were determined (see Table II).
In this study, we have biochemically characterized and determined the subcellular localization of the parasite 1-Cys peroxiredoxin TgTrx-Px2 and the 2-Cys peroxiredoxin TgTrx-Px1.

To analyze the antioxidant capacity of T. gondii thioredoxin-linked redox system, the biochemical parameters of TgTrx and TgTrx-Px1 were determined. It was established that TgTrx is a competent substrate for P. falciparum thioredoxin reductase, which emphasizes the structural similarity of the apicomplexan thioredoxins. Moreover, this similarity facilitated this study in that it allowed the use of PfTrxR. Additionally TgTrx is reduced by the dihydrodiolopamide dehydrogenase/dihydrodiolopamide reductase pair with a $k_\text{cat}$ of $1 \times 10^4 \text{M}^{-1} \text{s}^{-1}$, linking the thioredoxin redox to the diolopamide redox cycle. Although the thioredoxin under investigation is clearly a cytosolic protein the interaction with dihydrodiolopamide could play a role in the detoxification of hydroperoxides in the parasite organelles. Here lipoic acid is covalently attached to the α-keto acid dehydrogenase complexes and possibly can act as a reductant for a mitochondrial thioredoxin/peroxiredoxin-linked antioxidant defense system. This role has previously been suggested by Bunik (45) and Byrk et al. (46) and might also be relevant for T. gondii. TgTrx is also capable of reducing glutathione, with a $k_\text{cat}$ of $2.5 \times 10^2 \text{M}^{-1} \text{s}^{-1}$, similar to the value for PfTrx (28). Thus the different redox systems of T. gondii are highly integrated, which potentially is of great relevance in situations when the parasites are under enhanced oxidative stress.

TgTrx-Px1 has very low $K_v$ values for hydrogen peroxide and $t$-BuOOH suggesting that in vivo the enzyme is likely to be crucially involved in the reduction of these hydroperoxides. Notably, the catalytic efficiencies determined for the reduction of both hydroperoxides were between $2.2 \times 10^6 \text{M}^{-1} \text{s}^{-1}$ and $7.0 \times 10^5 \text{M}^{-1} \text{s}^{-1}$, which is 2–3 orders of magnitude higher than the catalytic efficiencies of peroxiredoxins of other organisms including trypanosomatids (1, 2, 47). They are, in fact, similar to those of catalases (2) and qualify the apicomplexan peroxiredoxin as an extremely efficient antioxidant. However, this high measured efficiency might be deceptive because TgTrx-Px1 is prone to substrate inhibition, probably by over-oxidation of its active site cysteine residue. TgTrx-Px1 has amino acid motifs GGIG at positions 94–97 and a tyrosine residue at position 192 that have been shown for other peroxiredoxins to be responsible for this susceptibility to overoxidation (6). This feature of eukaryotic 2-Cys peroxiredoxins explains their physiological roles beyond their peroxidase function and it has been suggested that they play an important part in redox signaling (4, 6, 48, 49). Possibly TgTrx-Px1 may well have such a role too.

The second class of the peroxiredoxin superfamily, the 1-Cys peroxiredoxins, are not as well studied as the 2-Cys peroxiredoxins. Only a small number of members of this protein class have been described and it is unclear what their precise role is in the antioxidant defense (3). The functionality of TgTrx-Px2 as thiol-specific-antioxidant was established using the GS protection assay (Fig. 4), which confirmed that the protein is a highly active peroxidase in the presence of dithiothreitol. However, it is not clear what its endogenous reducing partner is. A number of low molecular weight thiols and proteins including glutathione, glutaredoxin and cyclophilin have been suggested and recently interactions of 1-Cys peroxiredoxin with glutathione S-transferases and glutathione have been described (8, 9, 50). In this study, we tested the ability of glutathione, lipoic acid, thioredoxin, and glutaredoxin to reduce the TgTrx-Px2, but all four of them failed to act as electron donors for the protein. Therefore the question remains as to the role of this abundantly expressed protein and if it does reduce peroxides in vivo, as we have shown it can do in vitro, how does the cell return the protein to its reduced and active form?

Thus the data presented show that peroxiredoxin-linked detoxification of reactive oxygen species is a powerful cytosolic antioxidant system in T. gondii, helping the parasite to cope with the oxidative stress it encounters during its intracellular
life. However, despite the array of antioxidants, *T. gondii* tachyzoites are highly susceptible to exogenous and endogenous oxidative stress, with IC\(_{50}\) values for t-BuOOH, juglone, and phenazine methylsulfate in the nanomolar range (Fig. 1), and the strong DCF-fluorescence of oxidatively stressed *T. gondii* (Fig. 2). This suggests that minor changes in the redox balance of the parasites lead to destruction of their oxidant homeostasis and parasite death. Interestingly, the stressors appear to specifically affect the parasite and had no effect on the host cell. Thus interfering with the action of the peroxiredoxins might be a new avenue for the development of novel therapies against *T. gondii* and possibly also apicomplexan parasites in general.

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Susan E. Akerman and Sylke Müller

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